IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Gong et al.	Group Art Unit: 1632
Serial No.: 10/605,708	Examiner: Singh, Anoop Kumar
Filed: October 21, 2003	Atty. Dkt. No.: GLOF:007USC1
For: CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH	Confirmation No: 2707

DECLARATION OF ZHIYUAN GONG, PH.D.

I, Zhiyuan Gong, hereby declare as follows:

1. I am a co-inventor of the subject disclosed and claimed in the referenced application and I am familiar with the contents of said application. I am currently a professor of Biological Sciences, National University of Singapore, Singapore. I have extensive training and experience in the field of transgenic fish, as evidenced by my attached *curriculum vitae*, a copy of which is attached as Exhibit 1.

2. I understand that the PTO examiner in charge of examining the referenced patent application has rejected the pending claims, taking the position that the specification does not reasonably provide enablement for using any muscle specific promoter other than exemplified muscle specific promoters to obtain stable transgenic fish line suitable for ornamental fish marker showing fluorescence upon exposure to sunlight. I am submitting this declaration disagreeing on the enablement rejection for the following reasons.

3. The expression of identical transgenes that insert into different regions of a genome or a transgenic organism is variable, which is described as the "position effect." In this case the difference in expression is often due to local regulatory sequences that regulate neighboring genes or influences of local environment. Due to the position effect, a transgene, even under a strong promoter, will be expressed poorly, if at all, if it falls within a heterochromatin area or is silenced by a local regulatory sequence. To the contrary, a transgene, even under a weak promoter, could be expressed at a high level if its expression is enhanced by a local regulatory sequence, depending on the positional context.

4. It has been my experiences that a wide variation of expression is common for expressing transgenes in fishes, even where known strong promoters are employed to drive expression. Given the enormous number of potential sites of integration in a fish genome, expression of a transgene may be frequently affected by the position effect due to random integration of a transgene. Since not all transgenic constructs will behave in the manner that may be desired, a screening process for those fish with the desired level of transgene expression will be useful.

5. I have reviewed and am familiar with the specification of the referenced patent application, and would note that in Example III the specification teaches that one can screen the transgenic fish embryos to select those embryos exhibiting the desired expression characteristics. Particularly, preferred are those embryos exhibiting high expression such that the fluorescence is visible in the sunlight. I would also direct attention to Figures 8 through 12, particularly Figure 12, and their associated figure legends. The reason for using such an embryo selection technique (or any other suitable selection technique) is to identify a founder embryo that is likely

to give rise to a highly fluorescent line of fish that, if desired, express brightly enough to exhibit visible fluorescent coloration, visible even in sunlight.

6. Based on my knowledge and experience in the production of fluorescent, transgenic fish, it is my opinion that virtually any muscle-specific promoter can be employed to produce very highly fluorescent founder embryos and lines. While it may well be necessary in some instances to use one of the above or other screening procedure that permits one to select those embryos that have appropriate position effects, this should require only reasonably routine repetitive steps. Of course, when a weaker promoter is employed it may be necessary to inject and screen larger numbers of embryos, which may be more than one thousand, to identify a "high expresser" but again such screening is straightforward and does not involve any additional inventiveness to accomplish. Considering the muscle occupies a large part of the fish body and thus has the capacity to synthesize enough proteins for visible fluorescence, screening for visible fluorescence using any muscle-specific promoter provides specific guidance and predictable results for obtaining stable transgenic fish suitable for ornamental fish market.

7. I would further direct the examiner's attention to the attached article of Kinoshita entitled "Transgenic medaka with brillaint fluorescence in skeletal muscle under normal light" (*Fisheries Science, 70*:645-649, 2004). As the title implies, this article describes the preparation of transgenic, fluorescent medaka having a brilliant fluorescence in skeletal muscle under normal light. In these studies, the author employed the skeletal muscle actin promoter. Further, on page 648, col. 1, the author also mentions the article of Chou *et al.* (*Transgenic Res., 10*: 303-315, 2001), which is said to teach transgenic medaka strains with the GFP gene under the control of the β-actin gene regulatory region, which could be observed under daylight.

8. I further understand that in connection with the above-mentioned rejection, the examiner relies on a statement from page 62, col. 2, of my 2003 BBRC publication, with respect to which the examiner states that:

> It is clear from the teaching of Gong et al that strong expression of a fluorescent gene under the control of MLC2 promoter in muscle tissue that constitutes majority of the fish body tissue is vital for successfully generating transgenic fish for distribution in ornamental fish market.

This is not a true statement. As can be seen from reading the excerpt referred to by the examiner, it merely stands for the proposition that "one" consideration is the strength of the promoter, and that another consideration is the tissue specificity, with muscle promoters in general being preferred for this reason. However, nowhere does the article in any way state or imply that the MLC2 promoter is "vital" to producing our fluorescent transgenic fish. As explained above, we know that this particular promoter is not "vital" in this regard.

9. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

23/9/08
Date

Zhiyuan Gong

Exhibit 1

CURRICULUM VITAE

Name: **Zhiyuan Gong** Date: Dec. 2006
Date of Birth: April 6, 1959
Sex: Male

EDUCATION AND PROFESSIONAL EXPERIENCE:

July 2007--present. Professor, Department of Biological Sciences, National University of Singapore, Singapore

July 2006—June 2007, Associate Professor, Department of Biological Sciences, National University of Singapore, Singapore

July 2005-June 2006, Sabbatical leave at Department of Molecular, Cellular and Developmental Biology, University of California at Los Angeles, USA,

July 1997--June 2000. Senior Lecturer, Department of Biological Sciences, National University of Singapore, Singapore

June. 1995-- June 1997. Lecturer, Department of Biological Sciences, National University of Singapore, Singapore

Nov. 1988--June 1995. Research Fellow. Research Institute, Hospital for Sick Children, Toronto, and Departments of Biochemistry and Clinical Biochemistry, Banting Institute, University of Toronto.

Oct. 1987 -- Oct. 1988. Postdoctoral fellow, Department of Biology, McGill University, Montreal.

1984 (summer). Trainee in Embryology Course, Marine Biological Laboratory, Woods Hole, MA, U.S.A.

Sept. 1983 -- Oct. 1987. Department of Biology, McGill University, Montreal. **Ph.D. (1987), Dean's Honour List.**

Feb. 1982 -- Aug. 1983. Institute of Genetics and Department of Biology, Fudan University, Shanghai, China.

Feb. 1978 -- Jan. 1982. Department of Marine Biology, Ocean University of Qingdao (Shandong Oceanology College), Qingdao, China. **B.Sc. (1982)**

B. RESEARCH

I. MAJOR RESEARCH PROGRAMMES

Before Joining NUS

1. PH.D Project: Tubulin gene regulation in sea urchin embryos (1983-1987). I have demonstrated that tubulin genes are autogenously regulated by the concentration of free tubulin subunits in sea urchin embryos and the autogenous regulation plays an important role in developmental regulation of tubulin gene expression. This is the first demonstration of tubulin gene autoregulation in an in vivo system. The mechanism of the autoregulation is posttranscriptional at the level of RNA stability. In addition, I found that tubulin gene transcription could be stimulated by deciliation (removal of cilia). Five major papers have been published from this subject.

2. Postdotoral Research Projects (University of Toronto, 1988-1995)
In my postdoctoral training at University of Toronto, I was mainly involved in fish antifreeze protein gene project. While it was commonly believed that fish AFPs are produced exclusively in the liver and secreted into the blood for extracellular function, I found that the non-liver tissues also express AFP mRNAs in several species of fish and that non-liver AFP genes are regulated differently in response to the seasonal change and hypophysectomy. Furthermore, I have cloned several non-liver AFP genes and found that these non-liver genes encode AFPs without the signal peptide and prosequence which are commonly present in the liver AFPs. Therefore, the non-liver AFPs are likely to function intracellularly. This study opens a new area for antifreeze gene research in gene regulation and evolution. In addition, I was also involved in transgenic fish studies and cloning of pituitary hormone genes from several fish species.

Research Programs Developed in NUS

Since I joined NUS, I mainly used the zebrafish model for research. The zebrafish model was originally used for developmental analyses. To align with national R and D agenda, I also developed the zebrafish model for applications in biotechnology, environmental and medical sciences.

1. Zebrafish in Developmental Biology
Molecular dissection of neurogenic pathway in zebrafish embryos (NUS, since 1995). In the past few years, my laboratory has isolated many zebrafish cDNA clones involving in neural development. These cDNA clones belong to several gene families including *gli* zinc finger, *iroquois* homeobox, bHLH, and LIM homeobox families. By collaboration with Dr. V. Korzh, we have been actively characterizing their expression and function in developing zebrafish embryos and several mutants. Our current working hypothesis on the neurogenic pathway is: *sonic hedgehog --> gli/zic* zinc finger genes *-->iroquois*

homeobox genes --> bHLH genes --> LIM homeobox genes. While the work is still in progress, 12 papers have already published in major developmental biology journals such as *Development*, *Mechanism of Development* and *Developmental Dynamics*.

Zebrafish EST project (NUS, 1995-1998). In order to rapidly build up the genetic resources to facilitate molecular analyses in the increasingly important zebrafish model, our group was the first one to launch a zebrafish EST project for rapid isolation and identification zebrafish cDNA clones. Over the years from 1995 to 1998, we had generated over 3,000 zebrafish EST clones from several cDNA libraries including embryonic, whole adult, brain, eye, ovary and testis cDNA libraries. Our work was particularly important at the early stage of zebrafish EST project and helped to map the zebrafish genome before the initiation of the NIH funded zebrafish EST project in the large sequencing center in Washington University in 1998. During this period, we had received nearly 200 requests for zebrafish cDNA clones and libraries from all over the world. Recently, we have contributed two zebrafish gonad cDNA libraries (now named Gong Ovary and Gong Testis by the zebrafish community) to the international zebrafish EST project and over 25,000 EST clones were generated from these two libraries. 1,090 of these sequences have been used in the unigene set (16,000 genes) for designing of zebrafish microarray oligonucleotides by Compugen.

GFP transgenic zebrafish (NUS, since 1998). With the aid of our zebrafish EST project and an improved linker-mediated PCR method we developed, we were able to rapidly isolate gene promoters based on the EST clones. So far, we have isolated many tissue-specific and inducible promoters. These promoters have been linked with the GFP gene and introduced into zebrafish. We have generated dozens of stable transgenic lines for skin specificity, muscle specificity, liver specificity, exocrine pancreas specificity, oocyte specificity and neuron specificity. These transgenic lines are important assets for further analysis of promoters and gene expression programs, tracing cell lineage and migration, use for cell and nuclear transplantation etc. Some of the transgenic lines have two colors (GFP and RFP) for easy tracing development of multiple organs/tissues.

Evolutionary comparison of development of zebrafish swim bladder and tetrapod lung
As a member of SMA (Singapore-Massachusetts Institute of Technology), I proposed to work on development of zebrafish endodermal organs. The liver development is supported by the BMRC grant and the intestine organ is currently conducted by SMA students. Now I propose to work on swim bladder development. The swim bladder is a fish homolog of tetrapod lung. Comparison of development of swim bladder and lung will pose exciting questions not only in development but also in evolution. In embryos, both lung and swim bladder arise from an outgrowth of the gastrointestinal tract. The main function of the lung is to breathe air while the swim bladder can be inflated and deflated to change buoyancy in water. Furthermore, some lung-specific molecules such as surfactant proteins are also found in the swim bladder. Thus, the tetrapod lung and fish swim bladder are likely to share a common evolutionary origin. This proposal aims at understanding of the development and evolution of the two organs. In particular, we propose to compare expression pattern of important genes in lung and swim bladder development, and thus to identify the critical genes responsible for the distinction of developmental pathways for the two organs. The detailed characterization of fish swim

bladder, less vulnerable to infection, should also provide new insight into human lung development and lung infection.

Zebrafish in Biotechnology, Environmental and Medical Sciences

Fluorescent transgenic ornamental fish and transgenic fish bioreactor (NUS, since 1998). By strong expression of several fluorescent protein genes, including green, yellow and red fluorescent protein genes, in the skeletal muscle of zebrafish, we demonstrated that the fluorescent colors can be viewed by unaided eyes and thus it is feasible to use the transgenic technology to generate novel varieties of ornamental fish. Furthermore, by crossing with two transgenic lines of different color, we can generate new and intermediate colors, thus increasing the capacity of generating more transgenic colors. We also demonstrated that these transgenic fish can express recombinant proteins up to 17% of total muscle proteins, thus the muscle expression in transgenic fish may be used as a new transgenic bioreactor system. The technology for generation of fluorescent ornamental fish has been patented and licensed to an US company with a trademark **"GloFish".** The GloFish is being marketed in USA as the first genetically modified pet and received intensive attention worldwide, with a wide coverage in virtually all major global media outlets, including *CNN, BBC, CNBC, Fox, NHK, Newsweek, Nature, New Scientists, Washington Post New York Times, The Wall Street Journal, etc.*, with >500,000,000 media impressions (e.g. *Nature* **426**:372 and 596 [2003]; *Nature Biotechnology* **22**:1 [Editorial]; **22**:11 [2004]). It was also a debated topic in USA presidential election 2004 (*Nature.* 2004 Sep 16;431(7006):238-243). The significance of GloFish™ reaches far beyond the ornamental fish industry, as they have become a biotechnology showcase. In addition to their introduction into public aquaria for science education, GloFish™ have also been used as teaching materials in universities and high schools. As a biotechnology pioneer, GloFish™ will help the general public to establish faith in biotechnology products and will surely have a place in the history of biotechnology. Now the term GloFish has been included in Wikipedia, a web encyclopaedia, and *Singapore Encyclopaedia.* Because of our pioneer work, a new field of research is emerging by using our transgenic fish to evaluate the ecological effects of transgenic fish. Currently, we are also developing transgenic fish to express vaccines and to prove the concept of sushi-type edible vaccine.

Development of transgenic biomonitoring fish for environmental protection (NUS, since 1999). We have also generated transgenic biomonitoring fish using some inducible promoters to drive transgenic expression of fluorescent protein gene. Currently we are aiming at monitoring of endocrine disrupters and heavy metals. Both estrogen-responsive and heavy metal-inducible promoters have been isolated and engineered with the fluorescent protein genes. Stable transgenic lines have been developed for both zebrafish and medaka. Preliminary characterization of these transgenic fish indicated inducibility of GFP expression by many of the environmental relevant compounds. This work has made a finalist for Asian Innovation Award by *Far East Economic Review* (2002).

NEWater – Health effect studies using medaka fish as a model (Singapore national project, 1999-2003). Due to the shortage of water supply and the threat of cut-off from a neighbour country, Singapore government has launched a national water reclamation project using the membrane and reverse osmosis technology. The project was initiated in

1999 and I was appointed as the principle investigator by a national committee to conduct the fish study to test the health effect in reclaimed water (NEWater). In this project, we are using the medaka fish to conduct tumor and estrogenic tests. Because this is an unprecedented study, we have to develop all standard research protocols and all studies were carried out under stringent QA/QC monitoring. Two fish tests have been successfully conducted and a confidential report has been submitted through Ministry of Environment to the Prime Minister's Office for policy making.

Zebrafish liver program (NUS-BMRC project, since 2002). As the program coordinator and principle investigator, together with eight other co-PIs from DBS, Chemistry, Biochemistry, Genome Institute of Singapore and Institute of Molecular and Cell Biology, we have successfully won $5.3 BMRC (Biomedical Research Council of Singapore) co-operative research grant from the first round of national competition. In this project, we are focusing on molecular mechanisms of liver carcinogenesis and development using the zebrafish as a model. Other than coordinating the research activities among different groups across a few institutions/universities, I am leading my research to focus on development of zebrafish genomic tools, including generation of EST/full length cDNA sequences; production of zebrafish DNA chips using 60-mer oligonucleotides representing unigenes or singltons; investigation of zebrafish gene expression in response to environmental pollution and development of zebrafish DNA chip for environmental monitoring; generation of liver tumors by carcinogen treatment as well as by transgenic expression of oncogenes; and generation of liver and other tissue-specific GFP transgenic lines. By these studies, we wish to identify novel zebrafish genes involved in liver carcinogenesis and development. So far, we have published over 25 research papers under this program and, in particular, two of them appeared in top journals, *PLOS Genetics* and *Nature Biotechnology*. Our landmark works validated the zebrafish model for human disease studies by comparative analysis of transcriptome profiles in human and zebrafish liver cancers.

II. RESEARCH CONTRIBUTIONS:

A. Peer Reviewed Primary Research Papers published in international journals:

1. Floyd, E.E., **Z. Gong**, B.P. Brandhorst and W. H. Klein (1986) Calmodulin gene expression during sea urchin development: Persistence of a prevalent maternal protein. *Dev. Biol.* **103**:501-511.
2. **Gong, Z.** and B.P. Brandhorst (1987) Stimulation of tubulin gene transcription by deciliation of sea urchin embryos. *Mol. Cell. Biol.* **7**:4238-4246.
3. **Gong, Z.** and B.P. Brandhorst (1988) Autogenous regulation of tubulin synthesis via RNA stability during sea urchin embryogenesis. *Development* **102**:31-43.
4. **Gong, Z.** and B.P. Brandhorst (1988) Stabilization of tubulin messenger RNA by inhibition of protein synthesis in sea urchin embryos. *Mol. Cell. Biol.* **8**:3518-3525.
5. **Gong, Z.** and B.P. Brandhorst (1988) Multiple levels of regulation of tubulin gene expression during sea urchin embryogenesis. *Dev. Biol.* **130**:144-153.
6. **Gong, Z.** and B.P. Brandhorst (1988) Microtubule formation from maternal tubulins during sea urchin embryogenesis: Measurement of Soluble and insoluble tubulin pools. *Mol. Reprod. Dev.* 1:3-9.
7. **Gong, Z.**, P. Cserjesi, G.M. Wessel, and B.P. Brandhorst (1991) Structure and expression of the polyubiquitin gene in sea urchin embryos. *Mol. Reprod. Dev.* **28**:111-118.
8. **Gong, Z.**, C.L. Hew, and J.R. Vielkind (1991) Functional analysis and temporal expression of promoter regions from fish antifreeze protein genes in transgenic Japanese Medaka embryos. *Mol. Marine Biol. Biotech.* **1**: 64-72.
9. Lu, M., **Z. Gong**, W. Shen, and A.D. Ho (1991) The *Tcl*-3 proto-oncogene altered by chromosomal translocation in T-cell leukemia codes for a homeobox protein. *EMBO J.* **10**:2905-2910.
10. **Gong, Z.** (1992) Improved RNA staining in formaldehyde gels. *BioTechniques* **12**:74-76.

This paper has been selected with author's comments by *Rice Biotech.Quart.***11**:34.

11. Du, S.J., **Z. Gong**, G.L. Fletcher, M.A. Shears, M.J. King, D.R. Idler, and C.L. Hew (1992) Growth enhancement in transgenic Atlantic salmon by use of fish antifreeze/growth hormone chimeric gene constructs. *Bio/Technology* **10**:176-181.
12. Elsholtz, H.P., S. Majumdar-Sonnylal, F. Xiong, **Z. Gong** and C.L. Hew (1992) Phylogenetic specificity of prolactin gene expression with conservation of pit-1 function. *Mol. Endocrin.* **6**:515-522.
13. **Gong, Z.**, G.L. Fletcher and C. L. Hew (1992) Tissue distribution of fish antifreeze protein mRNAs. *Can. J. Zool.* **70**:810-814.
14. Du, S.J., **Z. Gong**, C.H. Tan, G.L. Fletcher and C.L. Hew (1992). The design and construction of "all fish" gene casette for aquaculture. *Mol. Marine Biol. Biotech.* **1**:290-300.
15. Iraqi, F., **Z. Gong**, L. Crim and C.L. Hew. (1993) Isolation and characterization of somatolactin genes from two cold water marine teleosts, lumpfish (*Cyclopterus lump*) and halibut (*Hippoglossus hippoglossus*). *Mol. Marine Biol. Biotech.* **2**: 96-103.

16. **Gong, Z.**, Z. Hu, Z. Q. Gong, R. Kitching, and C.L. Hew. (1994) Bulk isolation and identification of fish genes by cDNA clone tagging. *Mol. Marine Biol. Biotech.* **3**:243-251.
17. **Gong, Z.** and C.L. Hew. (1994) Zinc and DNA binding properties of a novel LIM domain homeobox protein Isl-2. *Biochemistry* **33**:15149-15158.
18. **Gong, Z.**, M.J. King, G.L. Fletcher, and C.L. Hew (1995) The antifreeze protein genes of the winter flounder, Pleuronectus americanus, are differentially regulated in liver and non-liver tissues. *Biochem. Biophys. Res. Commun.* **206**:387-392.
19. **Gong, Z.**, C.-c. Hui, and C.L. Hew (1995). Presence of *Isl-1* related LIM domain homeobox genes in teleost: their similar patterns of expression in brain and spinal cord. *J. Biol. Chem.* **270**: 3335-3345.
20. Wang, R., P. Zhang, **Z. Gong**, C.L. Hew (1995) The expression of antifreeze protein gene in transgenic goldfish (Carassius auratus) and its implication in cold adaptation. *Mol. Marine Biol. Biotech.* **4**: 20-26.
21. **Gong, Z.**, and C.L. Hew (1995) Several splicing variants of *isl-1* like genes in the chinook salmon (*Oncorhynchus tschawytscha*) encode truncated transcription factors containing a complete LIM domain. *Biochim. Biophy. Acta* **1260**:349-354.

Publications from NUS

1995

22. Tokumoto, M., **Z. Gong**, T. Tsubokawa, C.L. Hew, K. Uyemura, Y. Hotta, and H. Okamoto (1995) Molecular heterogeneity among primary motoneurons and within myotomes revealed by the differential mRNA expression of novel Islet homologs in embryonic zebrafish. *Dev. Biol.* **171**: 578-589.

1996

23. **Gong, Z.**, K.V. Ewart, Z. Hu, G.L. Fletcher, and C.L. Hew (1996) Skin antifreeze protein genes of the winter flounder, Pleuronectes americans, encode distinct and active polypeptides without the secretory signal and prosequences. *J. Biol. Chem.* **271**: 4106-4112.

1997

24. Liao, J., J. He and **Z. Gong** (1997) An abundant zebrafish cDNA clone encodes a ras-like protein which is expressed ubiquitously. *DNA Sequence* 7:313-317.
25. Liao, J. and **Z. Gong** (1997) Sequencing of 3' cDNA clones using anchored oligo dT primers. *BioTechniques* **23**:368-370.
26. **Gong, Z.**, T. Yan, J. Liao, S.E. Lee, J. He and C.L. Hew (1997) Rapid identification and isolation of zebrafish cDNA clones. *Gene* **201**:87-98.
27. He, J., Z. Yin, G. Xue, **Z. Gong**, T.J. Lam and Y.M. Sin (1997) Production of goldfish against *Ichthyophthrius multifilis* by immunization with a recombinant vaccine. *Aquaculture* **158**:1-10.
28. Liao, J., C.H. Chan and **Z. Gong** (1997) An alternative linker-mediated polymerase chain reaction method using a dideoxynucleotide to reduce amplification background. *Anal. Biochem.* **253**:137-139.

1998

29. Yan, T., and **Z. Gong** (1998) Assembly of a complete zebrafish mitochondrial 16S rRNA gene from overlapping expressed sequence tags. *DNA Sequence* 9:145-148..
30. Lim, J.H., J. He, V. Korzh and **Z. Gong** (1998) A new splicing variant of a type III POU gene from zebrafish encodes a POU protein with a distinct C-terminal. *Biochim. Biophy. Acta* **1397**:253-256.
31. Postlethwait, J.H., Y.-L. Yan, M.A. Gates, S. Horne, A. Amores, A. Brownlie, A. Donovan, E.S. Egan, A. Force, **Z. Gong**, C. Goutel, A. Fritz, R. Kelsh, E. Knapik, E. Liao, B. Paw, D. Ransom, A. Singer, M. Thomson, T.S. Abduljabbar, P. Yelick, D. Beier, J.-S. Joly, D. Larhammar, F. Rosa, M. Westerfield, L.I. Zon, S. Johnson and W. Talbot (1998) Vertebrate genome evolution and the zebrafish map. *Nature Genetics* **18**:345-349.
32. Korzh, V., I. Sleptsova, J. Liao, J. He, and **Z. Gong** (1998) Expression of zebrafish bHLH genes ngr1 and neuroD defines stages of an early neural differentiation. *Dev. Dynamics*. **213**:92-104.
33. Miao, M., S.-L. Chan, C.L. Hew and **Z. Gong** (1998) The skin-type antifreeze protein gene intron of the winter flounder is a ubiquitous enhancer lacking a functional C/EBPα binding motif. *FEBS Letters* **426**: 121-125.

1999

34. Xu, Y., J. He, H.L. Tian, C.H. Chan, J. Liao, T. Yan, T.J. Lam and **Z. Gong** (1999) Fast skeletal muscle specific expression of a zebrafish myosin light chain 2 gene and characterization of its promoter by direct injection into skeletal muscle. *DNA Cell Biol.* **18**:85-95.
35. Liao, J., J. He, T. Yan, V. Korzh and **Z. Gong** (1999) A class of NeuroD-related basic helix-loop-helix transcription factors which are expressed in developing central nervous systems in zebrafish. *DNA Cell Biol.***18**:333-344.
36. Xu, Y. and **Z. Gong** (1999) Adaptation of inverse PCR to generate an internal deletion. *Biotechniques*. **26**:639-641
37. Ju, B., Y. Xu, J. He, J. Liao, T. Yan, C.L. Hew, T.J. Lam and **Z. Gong**. (1999) Faithful expression of green fluorescent protein (GFP) in transgenic zebrafish embryos under zebrafish gene promoters. *Dev Genetics*. **25**:158-167.
38. Garg, R.R., L. Bally-Cuif, S.E. Lee, **Z. Gong**, X. Ni, C.L. Hew and C. Peng (1999) Cloning of zebrafish activin type IIB receptor (ActRIIB) cDNA and mRNA expression of ActRIIB in embryos and adult tissues. *Mol. Cell. Endocrinol.* **153**:169-181.
39. Dheen, T., I. Sleptova-Friedrich, Y. Xu, M. Clark, H. Lehrach, **Z. Gong** and V. Korzh (1999). Zebrafish *tbx2* plays a role in formation of the midline structures. *Development* **126**:2703-2713.
40. Tan, J.T.T., V. Korzh, and **Z. Gong** (1999). Expression of a zebrafish *iroquois* homeobox gene, *Ziro3,* in the midline axial structures and central nervous system. *Mech. Dev.* **87**:165-168.
41. Wang, H. and **Z. Gong** (1999). Characterization of two zebrafish cDNA clones encoding egg membrane proteins ZP2 and ZP3. *Biochim. Biophy. Acta.* **1446**:156-160.
42. Yin, Z, J.He, **Z. Gong**, T.J. Lam and Y.M. Sin (1999) Identification of differentially expressed genes in Con A-activated carp (*Cyprinus carpio* L.) leucocytes. *Comp Biochem Physiol B Biochem Mol Biol* **124**:41-50.

2000

43. Loh, S.H., W.T. Chan, **Z. Gong**, T.M. Lim, and K.L. Chua (2000) Characterization of a zebrafish (*Danio rerio*) desmin cDNA: an early molecular marker of myogenesis. *Differentiation* **65**:247-254.
44. Xu, Y., J. He, X. Wang, T. M. Lim and **Z. Gong** (2000) Asynchronous activation of 10 muscle specific protein (MSP) genes during zebrafish somitogenesis. *Dev. Dyn.* **219**:201-215.
45. Wang, X., H. Wan, V. Korzh and **Z. Gong** (2000) Application of an IRES bicistronic construct to trace the expression of exogenously introduced mRNA in zebrafish embryos. *Biotechniques* **29**:814-820.
46. Wang, H., T. Yan, J.T.T. Tan and **Z. Gong** (2000) A zebrafish vitellogenin gene (vg3) encodes a novel vitellogenin without a phosvitin domain and may represent a primitive vertebrate vitellogenin gene. *Gene* **256**: 303-310.
47. Gay, F., I. Anglade, **Z. Gong** and G. Salbert (2000) The LIM/homeodomain protein Islet-1 modulates estrogen receptor functions. *Mol. Endocrinol.* **14**:1627-48.

2001

48. Douglas, S.E., J.W. Gallant, **Z. Gong** and C. Hew (2001) Cloning and developmental expression of a family of pleurocidin-like antimicrobial peptides from winter flounder, *Pleuronectes americanus* (Walbaum). *Dev. Comp. Immunol.* **25**:137-147.
49. Sudha, P.M., S. Low, J. Kwang and **Z. Gong** (2001) Multiple tissue transformation in adult zebrafish by gene gun bombardment and muscular injection of naked DNA. *Marine Biotech.* **3**:119-125.
50. Wang, X., A. Emelyanov, I. Sleptova-Friedrich, V. Korzh, and **Z. Gong** (2001) Expression of two novel zebrafish Iroquois homologues (*ziro1* and *ziro5*) during devlopment of axial structures and central nervous system. *Mech. Dev.* **105**:191-195.
51. Chong, S.W., A. Emelyanov, **Z. Gong**, and V. Korzh (2001) Expression pattern of two zebrafish genes, *cxcr4a* and *cxcr4b*. *Mech. Dev.* **109**:347-354.
52. Wang, X., L.T. Chu, J. He, A. Emelyanov, V. Korzh and **Z. Gong** (2001) A novel zebrafish bHLH gene, neurogenin3, is expressed in the hypothalamus. *Gene* 275:47-55.

2002

53. **Gong, Z.**, B. Ju, X. Wang, J. He, H. Wan, P.M. Sudha and T. Yan (2002) Green fluorescent protein (GFP) expression in germ-line transmitted transgenic zebrafish under a stratified epithelial promoter from *keratin8* (*krt8*). *Dev. Dyn.* **223**: 204-215.
54. Venugopal, T., V. Anathy, T.J. Pandian, **Z. Gong** and S. Mathavan (2002). Molecular cloing of growth hormone encoding cDNA of an Indian major carp, Labeo rohita and its expression in E.coli and zebrafish. *Gen. Comp. Endocrinol.* **125**: 236-247.
55. Wan, H., J. He, B. Ju, T. Yan, T.J. Lam and **Z. Gong** (2002) Generation of two-color transgenic zebrafish using the green and red fluorescent protein reporter genes, *gfp* and *rfp*. *Marine Biotech.* 4: 146-154.
56. Zeng, S. and **Z. Gong**. (2002) EST analysis of expression profiles of zebrafish testis and ovary. *Gene* **294**: 45-53.
57. Wang, X., V. Korzh and **Z. Gong**. (2002) The functional specificity of NeuroD protein is defined by a single amino acid residue (N11) in the basic domain. *FEBS Lett.* **520**: 139-144.

58. Tan, C.H., T.C. Lee, S.D. Weeraratne, V. Korzh, T.M. Lim and **Z. Gong** (2002) *Ziwi*, the zebrafish homologue of the Drosophila *piwi*: co-expression with vasa at the embryonic genital ridge and gonad-specific expression in the adults. *Mech. Dev.* **119**:S221-S2244 (Also in *Gene Exp. Pattern* **2**:257-260).
59. Lam, S.H., H.L. Chua, **Z. Gong,** Z. Wen, T.J. Lam and Y.M. Sin (2002) Morphologic transformation of the thymus in developing zebrafish. *Dev. Dyn.* **225**:87-94.

2003

60. Ju, B., S.W. Chong, J. He, X. Wang, Y. Xu, H. Wan, Y. Tong, T. Yan, V. Korzh and **Z. Gong** (2003) Recapitulation of fast skeletal muscle development in zebrafish by transgenic expression of GFP under the *mylz2* promoter. *Dev. Dyn.* **227**:14-26.
61. Irwin, D.M. and **Z. Gong** (2003) Molecular evolution of vertebrate goose-type lysozyme genes. *J. Mol. Evol.* 56:234-242.
62. Wang, X., A. Emelyanov, V. Korzh and **Z. Gong** (2003) Zebrafish atonal homologue *zath3* is expressed during both neurogenesis and gliogenesis. *Dev Dyn.* **227**:587-92.
63. **Gong, Z.**, H. Wan, T.L. Tay, H. Wang, M. Chen and T. Yan (2003) Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle. *Biochem. Biophys. Res Comm.* 388:58-63.

2004

64. Lam, S.H., H.L. Chua, **Z. Gong,** T.J. Lam and Y.M. Sin (2004) Development and maturation of the immune system in zebrafish, Danio rerio: A gene expression profiling, in situ hybridization and immunological study. *Dev. Comp. Immunol.* **28**:9-28.
65. Tong, Y., T. Shan, Y.K. Poh, T. Yan, H. Wang, S.H. Lam and **Z. Gong** (2004) Molecular cloning of zebrafish and medaka vitellogenin genes and comparison of their expression in response to 17β-estradiol (E2). *Gene* **328**:25-36.
66. Mudumana, S.P., H. Wan, M. Singh, V. Korzh and **Z. Gong** (2004) Expression analyses of zebrafish *transferrin*, *ifabp* and *elastaseB* mRNAs as differentiation markers for the three major endodermal organs: liver, intestine and exocrine pancreas. *Dev. Dyn.* **230**:165-173.
67. Wu, Y.L. and **Z. Gong** (2004) A Novel Zebrafish Kelch-like Gene *klhl* and its Human Ortholog *KLHL* Display Conserved Expression Patterns in Skeletal and Cardiac Muscles. *Gene* **338**:75-83.
68. Lam, S.H., S. Mathavan, Y. Tong, J. Hu, C.L. Winata, S. Lee, L.D. Miller, E.T. Liu, and **Z. Gong** (2004) Preliminary microarray analyses of gene expression in zebrafish treated with xenobiotic and bioactive compounds. *Marine Biotech.* **6**: S468-S474.

2005

69. Ke, Z., A. Emelyanov, S.E.S. Lim, V. Korzh, **Z. Gong** (2005) Expression of a novel zebrafish zinc finger gene, *gli2b*, is affected in Hedgehog and Notch signaling related mutants during embryonic development. *Dev. Dyn.* **232**:479-486.
70. Pan, X., H. Wan, W. Chia and **Z. Gong** (2005) Demonstration of site-directed recombination in transgenic zebrafish using the Cre/loxP system. *Transgenic Research* 14: 217 – 223.

71. Lam, S.H., Y.M. Sin, **Z. Gong** and T.J. Lam (2005) Effects of thyroid hormone on the development of immune system in zebrafish. *Gen. Comp. Endocrinol.* 142:325-335.
72. Wang, H., J.T.T. Tan, A. Emelyanov, V. Korzh and **Z. Gong** (2005) hepatic and extrahapatic expression of vitellogenin genes in the zebrafish *Danio rerio. Gene* 356:91-100.
73. Zeng, Z., X. Liu, S. Seebah and **Z. Gong** (2005) Faithful expression of living color reporter genes in transgenic medaka under two tissue-specific zebrafish promoters. *Dev. Dyn.* 234:387-392.
74. Zeng, Z., T. Shan, Y. Tong, S.H. Lam and **Z. Gong** (2005) Development of estrogen-responsive transgenic medaka for environmental monitoring of endocrine disrupters. *Env. Sci. Tech.* 39:9001-9008.
75. Mathavan, S., S.G.P. Lee, A, Mak, L.D. Miller, K.R.K. Murthy, K.R. Govindarajan, Y. Tong, Y.L. Wu, S.H. Lam, H. Yang, Y. Ruan, V. Korzh, **Z. Gong**, E.T. Liu and T. Lufkin (2005) Transcriptome analysis of zebrafish embryogenesis using microarrays. *PloS Genetics* 1: 260-276.

2006

76. Zhu, S, L. Liu, V. Korzh, **Z. Gong** and B.C. Low (2006) RhoA acts downstream of Wnt5 and Wnt11 to regulate convergence and extension movements by involving effectors Rho Kinase and Diaphanous: use of zebrafish as an in vivo model for GTPase signalling. *Cellular Signalling* 18:359-372..
77. Lam, S.H, Y.L. Wu, V.B. Vega, L.D. Miller, J. Spitsbergen, Y. Tong, H. Zhan, K.R. Govindarajan, S. Lee, S. Mathavan, D.R. Buhler, E.T. Liu, and **Z. Gong** (2006) Conservation of gene expression signatures between zebrafish and human liver tumors and tumor progression. *Nature Biotechnology* 24: 73-75. (Publication with eight supplementary files)

A News and Views article by Grabher and Look was published in the same issue of *Nature Biotechnology* to comment on the significance of this work.

78. Lam, S.H. and **Z. Gong** (2006) Modelling Liver Cancer Using Zebrafish: A Comparative Oncogenomics Approach. *Cell Cycle* 5:573-577.
79. Tay, T.L, Q. Lin, T. K. Seow, K. H. Tan, C. L. Hew and **Z. Gong** (2006) Proteomic analysis of protein profiles during early development of the zebrafish, *Danio rerio. Proteomics*, 6:3176-3188.
80. Wan, H., S. Korzh, Z, Li, S.P. Mudumana, V. Korzh Y.-J. Jiang, S. Lin and **Z. Gong** (2006) Analyses of pancreas development by generation of *gfp* transgenic zebrafish using an exocrine pancreas-specific *elastaseA* gene promoter. *Exp. Cell Res.* 312:1526-1539.
81. Liu, X., H. Wang and **Z. Gong** (2006) Tandem-repeated Zebrafish *zp3* Genes Possess Oocyte-specific Promoters and Are Insensitive to Estrogen Induction. *Biology of Reproduction* 74:1016-1025.
82. Huang, W.D., Z. Lin, Y.M. Sin, D. Li, **Z. Gong** and D. Yang (2006) Characterization and expression of a cDNA encoding a tubuliform silk protein of the golden web spider Nephila antipodiana. *Biochime.* 88:849-858.
83. Lam, S.H., C.L. Winata, Y. Tong, S. Korzh, W.S. Lim, V. Korzh, J. Spitisbergan, S. Mathavan, L.D. Miller, E.T. Liu and **Z. Gong** (2006) Trancriptome Kinetics of Arsenic-induced Adaptive Response in Zebrafish Liver. *Physilogical Genomics.* 27:351-361.

2007

84. Dong, P.D.S., C.A. Munson, W. Norton, C. Crosiner, X. Pan, **Z. Gong**, C.J. Neumann and D.Y.R. Stainier (2007) Fgf10 regulates hepatopancreatic ductal system patterning and differentiation. *Nature Genetics* 39:397-402.
85. Song, J., H.J. Kim, **Z. Gong**, N.-A. Liu and S. Lin (2007) *Vhnf1* acts downstream of Bmp, Fgf, and RA signals to regulate endocrine beta cell development in zebrafish. *Dev. Biol.* 303:561-575.
86. Zhu, S., L. Liu, V. Korzh, **Z. Gong** and B.C. Low (2007) RhoA prevents apoptosis during zebrafish embryogenesis through activation of Mek/Erk pathway. Oncogene. 27:1580-1589..
87. Li. Z., V. Korzh and **Z. Gong** (2007) Localized rbp4 expression in the yolk syncytial layer plays a role in yolk cell extension and early liver development. BMC Dev Biol. 7:117 [Epub ahead of print].
88. Wu, Y.L., X. Pan, H. Wang, P.W. Kee, S.P. Mudumana and **Z. Gong** (2007) Development of a heat shock inducible gfp transgenic zebrafish line by using the zebrafish hsp27 promoter. Gene. 408:85-94.
89. Ke, Z., I. Kondrichin, **Z. Gong** and V. Korzh (2007) Combined activity of the two Gli2 genes of zebrafish play a major role in Hedgehog signaling during zebrafish neurodevelopment. Mol Cell Neurosci. 37: 388-341.

2008

90. Hegde A, Qiu NC, Qiu X, Ho SH, Tay KQ, George J, Ng FS, Govindarajan KR, Gong Z, Mathavan S, Jiang YJ. (2008) Genomewide expression analysis in zebrafish mind bomb alleles with pancreas defects of different severity identifies putative Notch responsive genes. PLoS ONE. 3(1):e1479.
91. Farooq M, Sulochana KN, Pan X, To J, Sheng D, Gong Z, Ge R. (2008) Histone deacetylase 3 (hdac3) is specifically required for liver development in zebrafish. Dev Biol. 317:336-353.
92. Pan X, Zhan H, Gong Z. (2008) Ornamental Expression of Red Fluorescent Protein in Transgenic Founders of White Skirt Tetra (Gymnocorymbus ternetzi). *Mar Biotechnol* 10:497-501.
93. Asharani, P.V., Y.L. Wu, Z. Gong and S. Valiyaveettil (2008) Toxicity of silver nonoparticles in zebrafish model. *Nonotechnology* 19:255102.
94. Asharani, P.V., N.G.B. Serina, M.H. Numawati, Y.L. Wu, Z. Gong and S. Valiyaveettil (2008) Impact of multi-walled carbon nanotubes on aquatic species. *J. Nonosci. Nonotech.* 8: 3603-3609.
95. Lam, SH, Mathavan S, Tong Y, Li H, Karuturi RKM, Wu YL, Vega VB, Liu ET and Gong Z. (2008) Zebrafish Whole-Adult-Organism Chemogenomics for Large-Scale Predictive and Discovery Chemical Biology. PLOS Genetics. 4:e1000121.
96. Liu, L., S. Zhu, Z. Gong and B.C. Low (2008) K-ras/PI3K-Akt signaling is essential for zebrafish hematopoiesis and angiogenesis. *Plos One* 3:e2850.
97. Liu, X., Z. Li, A. Emelyanov, S. Parinov and Z. Gong (2008) Generation of oocyte-specifically expressed cre transgenic zebrafish for female germline excision of loxP-flanked transgene. *Dev. Dyn.* In press.
98. Li, Z., C. Wen, J. Peng, V. Korzh and Z. Gong (2008) Generation of living color transgenic zebrafish to trace somatostatin-expressing cells and endocrine pancreas organization. *Differentiation* In press.

B. Invited Reviews, Monographs and Book Chapters

1. Hew, C.L., S.J. Du, **Z. Gong**, G.L. Fletcher, and P.L. Davies (1991) Biotechnology in aquatic sciences: improved freezing tolerance and enhanced growth in Atlantic salmon by gene transfer. *Bull. Insti. Zool., Academia Sinica* (Taipei). 16:341-356.
2. Hew, C.L., and **Z. Gong** (1992) Transgenic fish: a new technology for fish biology and aquaculture. *Biology International* **24**:2-10.
3. Du, S.J., **Z. Gong**, G.L. Fletcher, M.A. Shears, and C.L.Hew (1992) Growth hormone gene transfer in atlantic salmon: use of fish antifreeze/growth hormone chimeric gene constructs and application of polymerase chain reaction. in "Transgenic Fish", C.L. Hew and G.L. Fletcher, eds. pp.176-189.
4. **Gong, Z.** and C.L. Hew (1993) Promoter analysis of fish antifreeze protein genes. *Biochemistry and Molecular Biology of Fishes*, volume 2, (P.W. Hochachka and T.P. Mommsen, eds) pp.307-324.
5. Xiong, F., R. Chin, **Z. Gong**. K. Suzuki. R. Kitching, S. Majumdar-Sonnylal, H.P. Elsholtz and C.L. Hew (1993) Control of salmon pituitary hormone gene expression. *Fish Physiol. Biochem.* **11**:63-70.
6. Hew, C.L., S.J. Du, **Z. Gong**, P.L. Davies, S.Y. Gauthier, M.A.Shears, M.J. King and G.L. Fletcher. (1993) Transgenic salmon with enhanced growth and freeze resistance. *Biology International* **28**:87-94.
7. **Gong, Z.**, and C.L. Hew (1995) Transgenic fish in aquaculture and developmental biology. *Current Topic Dev. Biol.* **30**:177-214.
8. **Gong, Z.**, S.J. Du, G.L. Fletcher, P.L. Davies and C.L. Hew (1995) Application of transgenic fish technology in aquaculture. Proceedings of the International Symposium on Biotechnology Applications in Aquaculture, C.-M. Kuo, J.-L. Wu, and P.-P. Hwang, eds. pp.41-54.
9. Hew, C.L., **Z. Gong**, S.J. Du, M.A. Shears, M.J. King, G.L. Fletcher, P.L. Davies, and R. Saunders (1998) Use of the fish antifreeze protein gene promoter in the production of growth hormone-transgenic salmon with enhanced growth performance. In: Biotechnology in Agriculture. A. Altman (ed) Marcel Decker, Inc., NY. pp.549-561.
10. Fletcher, G.L., S.V. Goddard, P.L. Davies, **Z. Gong**, K.V. Ewart and C.L. Hew (1998) New insights into fish antifreeze proteins: physilogical significance and molecular regulation. In: Cold Ocean Physiology. H.O. Portner and R.C. Playle (eds). Cambridge University Press.
11. **Gong, Z.** (1999) Zebrafish expressed sequence tags and their applications. *Methods Cell Biology* **60**:213-233.
12. **Gong, Z.**, P.M. Sudha, B. Ju, Y. Xu, J. He, H. Wang, S. Zeng, H. Wan, X. Wang and T. Yan (1999) Applications of the transgenic technique to fish and shrimps. *J. Ocean Univer. Qingdao* 29:649-657.
13. Anglade, I, S. Planchot, **Z. Gong**, C.L. Hew, O. Kah and G. Salbert (1998) Distribution of islet-expressing cells in the forebrain and pituitary of the rainbow trout. *Ann NY Acad. Sci.* **839**:420-421.
14. **Gong, Z.**, B. Ju and H. Wan (2001) *Green fluorescent protein* (*GFP*) transgenic fish and their applications. *Genetica* **111**:213-225.

15. Melamed, P., **Z. Gong**, G.L. Fletcher, and C.L. Hew, (2002) The impact of biotechnology in aquaculture. *Aquaculture* **204**:255-269
16. **Gong, Z.**, H. Wan, B. Ju, J. He, X. Wang and T. Yan (2002) Generation of living color transgenic zebrafish. In: Aqua Genome: Steps toward a Great Future. N. Shimizu, T. Aoki, I Hirono, F. Takashima (eds). Pp.329-339.
17. **Gong, Z.**, H. Wan, M. Chen and T. Yan (2002) Applications of transgenic technology in ornamental fish. *Fisheries Sci.* **68**:1063-1066.
18. **Gong, Z.**, Y.L. Wu, S.P. Mudumana and S. Lin (2004) Transgenic fish for developmental biology studies. In: Fish Development and Genetics: the zebrafish and medaka model. Z. Gong and V. Korzh (eds). World Scientific. Pp.476-516.
19. Low, B.C. and **Z. Gong** (2005) Reporter Gene System: Green Fluorescent Protein. In: Encyclopedia of Molecular Cell Biology and Molecular Medicine. 2nd Edition. R.A. Meyers (ed). Wiley-VCH. Volume 12: 215-248.
20. **Gong, Z.**, N. Maclean, R.H. Devlin, R. Martinez, O. Omitogun and M. P. Estrada (2007) Chapter 4: Gene Construct and Expression: Information Relevant for Risk Assessment and Management. In: Environmental Risk Assessment of Genetically Modified Organisms: Methodologies for Transgenic Fish (eds; A. Kapuscinski, S. Li and K. Hayes).
21. Kapuscinski, A., G. Dana, K. Hayes, S. Li, K. Nelson, Y. K. Nam, **Z. Gong**, R. Devlin, G. Mair and W. Senanan (2007) Chapter 10: Risk Assessment of Transgenic Fish: Synthesis and Conclusions. In: Environmental Risk Assessment of Genetically Modified Organisms: Methodologies for Transgenic Fish (eds; A. Kapuscinski, S. Li and K. Hayes).

C. Book

1. **Gong, Z.** and V. Korzh (2004) Fish Development and Genetics: the zebrafish and medaka models. World Scientific. PP. 675.

4. Research Grants:

University research grants:

1. PI. Academic Research Fund, $223,335, 1995-1998: Developmental regulation and functional analysis of a family of LIM domain homeobox genes in zebrafish.

2. PI, Academic Research Fund, $70,330, 1995-1997: Identification of surface antigens in *Ichthyophthirius multifiliis* and the development of fish vaccine.

3. PI. Academic Research Fund, $114,550, 1996-1999: Generation of novel varieties of ornamental fish by transgenic expression of green fluorescent protein (GFP).

4. Collaborator. Academic Research Fund, $151,200, 1996-1999: Sex differentiation in fish (PI, A/P Tan Choong Huat).

5. PI. Academic Research Fund, $245,000, 1998-2002: Molecular dissection of neurogenic pathway in zebrafish.

6. PI, Academic Research Fund, $74,550, 1999 -2003: Production of fluorescent transgenic ornamental fish.

7. PI, Academic Research Fund, $175,395, 1999 - 2003: Transgenic expression of spider silk protein genes in the silkworm, *Bombyx mori*.

8. Collaborator. Academic Research Fund, $73,250, 2000-2003: Gonadogenesis during early development of fish embryos. (PI. A/P Tan Choong Huat)

9. Collaborator, Academic Research Fund, $86,085, 2000-2003: Development of the immue system in fish larvae and its possible control by thyroid hormones. (PIs: Prof. Lam Toong Jin and A/P Sin Yoke Min)

10. Collaborator, Academic Research Fund, $63,069, 2001-2003: Sextual behaviour, reproduction and viability of fluorescent transgenic zebrafish. (PI, Dr. Li Daiqin)

Research projects funded by government agencies or industry:

1. PI, ENV/PUB/NSTB Newater project for fish test and research 1999-2002 (Phase I): budgeted, $620,000; secured, $461,850. Fish test in Water Quality Program under National Water Reclamation (NEWater).

2. Co-PI, NSTB grant 2001-2004, Establishment of a lab of excellence in aquatic and marine biotechnology (LEAMB) (S$1.5M, shared with four other groups, PI, Prof. Hew Choy Leong)

3. Co-PI of toxicogenomics in BFIG-II (Biosensor Focus Group, Phase II). Funded by NSTB/NUS (2001-2003). (PI, Prof. P. Gopalakrishnakone)

4. PI, ENV/PUB/NSTB Newater project, $228,000; 2002 -2003 (extension): Fish test in Water Quality Program under National Water Reclamation (NEWater).

5. PI, (co-PIs: Drs. B.C. Low, V. Korzh, E. Liu, S. Mathavan, R. Ge, W.K. Chan, T.P. Loh and R. Shoba) BMRC (Biomedical Research Council of Singapore) co-operative grant 2002-2007, $5,354,970. Molecular mechanisms of liver development and hepatocarcinogenesis: the zebrafish model.

6. Co-PI (PI, Dr. Jiang Yun-Jin) A*STAR BMRC-JDRF GRANT: Notch signalling and control of stem cell differentiation in zebrafish pancreas (04/1/50/22/295), 2004, S$248,750 for 2 years (11/12/04-10/12/06).

7. PI (co-PI, Drs. Ong Chone Nam, Lam Siew Hong, Xie Rongjing, Zhang Lifeng, S. Mathavan, E. Liu) EWI (Environment and Water Industrial Council), 2007-2010. $1,699,000. Use of Small Aquarium Fish to Develop Biological Monitoring Systems for Water Quality and Security.

8. PI (collaborators: V. Korzh, A. Ip, Hew CL) MOE Tier 2. 2007-2010. $632,600. Molecular Characterization of Fish Swimbladder Development: Implications of the Origin and Evolution of Lung in Tetrapods

5. Membership of institutional, national or international scientific advisory boards

1. Board member, IUBS-RBA (International Union of Biological Sciences--Reproductive Biology in Aquaculture), since June 1997.
2. Elected member of Asia-Pacific IMBN (International Molecular Biology Network), since Jan. 2001.
3. Scientific Advisory Board of Yorktown Technologies, USA, the GloFish company. My role is as an inventor of GloFish™ to provide expert opinion on development of new varieties of transgenic GloFish™ and to evaluate their environmental impact for USA government regulatory purpose. Since 2002
4. Committee member of International Society of Aquatic Genomics, since 2003.
5. Invited member of GMAC for reviewing transgenic fish, Singapore. 2003
6. Invited member of Advisary Board of Science Center, Singapore. Since 2003

6. Membership of Editorial Boards

1. Co-editor of Molecular Aspects of Fish and Marine Biology, World Scientific (since 1999). So far, four volumes of monographs have been published.

2. **Assistant Editor of *Journal of Fish Biology*** (since Jan 2006). My role is to conduct review and revision of manuscripts on fish molecular biology and to make recommendation of acceptance or rejection of the manuscripts.

10. Invited speakers at conference/symposia

Prior to NUS appointment (1993-1995)

1. Invited Speaker, "The LIM domain homeobox gene isl-1 in salmon". Taniguchi Symposium on Developmental Biology V: Gene Regulation in Development of Aquatic Animals. Qingdao, China. April 1993.
2. Invited Instructor of IUBS workshop on Reproductive Biology and Agriculture, Qindao, China, April 1993
3. Invited speaker, "From gene tagging to transciption factors". IUBS Symposium: Advances in the Molecular Endocrinology of Fish. Toronto. May 1993.
4. Invited speaker, "Application of transgenic fish technology in aquaculture" . International Symposium on Biotechnology Applications in Aquaculture, Taipei, Taiwan. Dec. 1994.
5. Invited speaker, "A family of novel LIM homeodomain proteins from teleosts: molecular characterization, expression analysis and DNA binding properties". Workshop on LIM Proteins and the LIM Domain, Bischenberg, France, May 1995.

Representing NUS (since 1996)

1996

6. Invited Plenary Keynote Speaker, "Transgenic fish and marine biotechnology" Asia-Pacific Conference on Science and Management of Coastal Environment, Hong Kong, June 25-28, 1996.

1997

7. Invited Speaker, "Sequence tag project in the zebrafish" in Current Advances in Defining the Zebrafish Genome, Boston, MA, U.S.A. Feb. 2-4, 1997.
8. Invited Session Chair and Speaker, "Zebrafish neuroD, a potential upstream gene of the neuroendocrine transcription factor Isl-1". 2nd IUBS Toronto Symposium "Advances in the Molecular Endocrinology of Fish" May 16-19, 1997, Toronto, Canada.
9. Invited Session Chair and Speaker, "Massive cloning of zebrafish genes and their applications". 7th SCBA International Symposium. July 6-11, 1997, Toronto, Canada.

1998

10. Invited Keynote Speech, "Massive cloning of fish genes and their applications in transgenic fish". International Symposium on Progress and Prospect of Marine Biotechnology (ISPPMB'98), Oct. 6-9, 1998, Qingdao, China.
11. Invited Speaker, "Application of transgenic techniques in fish and shrimp diseases" UNESCO workshop on shrimp disease, Oct. 9-14, 1998, Qingdao, China.

1999

12. Invited Session Chair and Speaker, "From zebrafish EST clones to transgenic ornamental fish". Aquarama'99: World Conference on Ornamental Fish Aquaculture. June 3-6, 1999, Singapore.

2000

13. Invited Plenary Keynote Speaker, "Application of transgenic technology in aquaculture and developmental biology". IMBC 2000: International Marine Biotechnology Conference. Sept. 28-Oct. 4, 2000, Townsville, Austrilia.
14. Invited Principal Speaker, "Generation of living color transgenic zebrafish". International Symposium: A Step Toward the Great Future of Aquatic Genomics. Nov. 10-12, 2000, Tokyo, Japan.
15. Invited Speaker. "Application of transgenic technology in aquaculture". 7th Pacific Rim Biotechnology Conference and Bioexpo 2000. Nov. 12-16, Vancouver, Canada.
16. Invited Speaker. "Generation of living color transgenic zebrafish" International Symposium on Marine Biotechnology, Dec. 6-8, 2000, Qindao, China.

2001

17. Invited Speaker, "Generation of living color transgenic zebrafish". International Conference on Advanced Technologies in Fisheries and Marine Sciences. Feb. 2-4, 2001, Tamil Nadu, India.
18. Invited Speaker, "Generation of living color transgenic ornamental fish". Aquarama: 2nd World Conference on Ornamental Fish Aquaculture "Modern Technology for the Future". May 31-June 2, 2001, Singapore.
19. Invited Speaker, "Applications of transgenic technology in ornamental fish". 70th Anniversary of the Japanese Society of Fisheries Science: International Commemorative Symposium. Oct. 1-5, 2001. Yokohama, Japan.
20. Invited Speaker and session chair, "Generation of rainbow color transgenic zebrafish". The Sixth Asian Fisheries Forum. Nov. 25-30, 2001, Kaohsing, Taiwan. (unable attend due to an urgent mission in the national NEWater project).

2002

21. Invited Speaker. "Generation of rainbow color transgenic zebrafish" World Aquculture 2002. April 23-27, 2002. Beijing, China. (unable to attend due to teaching duty, but the paper was presented by my student, Mr. Wan Haiyan, on my behalf).

2003

22. Invited Speaker, "Transgenic fish technology" 7th International Symposium on Reproductive Physiology of Fish. May 18-23, 2003, Mie, Japan. (unable to attend because of conduct of re-sit examination)
23. Invited Speaker, "Promises of transgenic fish biotechnology" Seminar on current Advances in Biotechnology. June 6, 2003. Jakarta, Indonesia.
24. **Invited Speaker, "Application of transgenic technology to ornamental fish" XIX International Congress of Genetics. July 6-11, 2003. Melbourne, Australia.**

Our research was highlighted in the Congress' daily briefing. Following my report I the Congress, I was interviewed by many world-wide media including *Scientific America, New Scientists* and many Australian media and our research story had been published in many Australia newspapers and radios during the Congress.

25. Invited Speaker, "Transgenic fish for ornamentals and bioreactors" AusBiotech2003. Aug. 16-19, Adelaide, Australia.
26. Invited Keynote Speaker, "Transgenic fish for ornamentals and bioreactors". 6th International Marine Biotechnology Conference. Sept. 21-24, 2003. Chiba, Japan.

27. Invited Speaker, "Zebrafish DNA chip" 2nd Aquatic Genomic Conference, Sept. 25-27. 2003. Tokyo, Japan.
28. Invited Speaker, "transgenic fish" Biosafety Science of Genetically Engineered Organisms. Oct. 27-28, 2003. Chonburi, Thailand.
29. Invited Speaker, "Application of transgenic technology to ornamental fish" The 8th International Aquarium Fish & Accessories Exhibition & Conference, Oct 30 – Nov. 2, 2003, Singapore.

2004

30. Invited Speaker, "Transgenic fish for ornamentals, bioreactors, vaccines and biomonitoring". World Aquaculture 2004, March 1-4, 2004, Honolulu, USA.
31. Invited Public Lecturer, "Future of transgenic fish". Singapore International Fish Show. March 12-15, 2004, Singapore.
32. Invited speaker, on aquaculture biotechnology. Indo-Singapore Joint Workshop on Aquaculture and Marine Biotechnology, April 22-24, Kochi, India. (did not attend because of university exam).
33. Invited Speaker, Exploration and Application of Marine Gene Resources. May 18-20. Beijing, China. (did not accept)
34. Invited Speaker, "Zebrafish model in human diseases" Sir Edward Youde Memorial Fund Postgraduate Conference 2004-Model Organism Research and Human Diseases. June 14-15, 2004, Hong Kong.
35. Invited Speaker, "China International Recreation Fisheries and Aquaria 2004 Conference" Sept. 9-12, 2004, Guangzhou, China. (did not accept because of teaching duty).
36. Invited Speaker, "Of fish and chips: Genome-wide expression profiling studies using a zebrafish DNA chip". Fish Genetics and Development. Oct. 11-14, 2004. Wuhan, China.
37. Invited Speaker, "Genome-wide expression profiling studies using a zebrafish DNA chip" Asian/Oceanian fish meeting, Nov. 15-16, 2004, RIKEN, Kobe, Japan.
38. Invited Speaker, Sixth Asia-Pacific Marine Biotechnology Conference: Unique Processes and Novel Products, Nov. 28-Dec. 2, 2004, Zhoushan, China (did not accept)
39. Invited Speaker, 1. "DNA microarray technology in fish"; 2. "Transgenic fish technology". Workshop on "Genomics and Its Related Techniques Applied to Aquatic Organisms". Dec. 17-22, 2004, Shanghai, China.
40. Invited Keynote Speaker, "Application of transgenic fish technology". 1st COE International Symposium on "Potential and Perspective of Marine Bio-Manipulation" Feb. 26-27, 2004, Sapporo, Japan

2005

41. Invited Speaker, "A fish model in environmental monitoring and cancer research: the DNA microarray approach", Workshop on Pearl Oyster Genome and Application. March 23, 2005, Haikou, China,
42. Invited Speaker, International Symposium on Genetically Modified Organisms. Sept 2-3, 2005, Jeju City, Korea (did not make it because of sabbatical leave)
43. Invited Participant and Chapter Leader. United Nations Environmental Programme STAP (Scientific and Technical Advisory Panel) book writing workshop on the

environmental risk assessment of transgenic fish. 17-21 October 2005, Penang, Malaysia.

44. Invited Speaker. International Congress "Biotechnology Havana 2005", Nov. 27-Dec. 2, 2005, Havana, Cuba. (did not make it because of sabbatical leave)

2006

45. Invited Speaker, "Promising applications of transgenic fish technology in aquaculture, environmental monitoring and cancer biology". 2nd Norwegian Transgenic Animal Forum, March 23-24, 2006, Vikersund, Norway.

2007

46. Invited Speaker, "Asian biotech aquaculture and the perspectives of Asian governments on sustainability" (topic given by the organizer) Aquaculture America '07. February 26 to March 2, 2007, San Antonio, Taxes, USA.
47. Invited Speaker, "Zebrafish as a human disease model" (tentative) Model Systems for Infectious Disease and Cancer in Zebrafish, Jul 16-18, 2007, Amsterdam, Holland.
48. Invited Speaker, "Applications of transgenic fish and fish DNA chips in environmental monitoring". International Symposium on Biotechnology in Agriculture. July 26-27, 2007. Qingdao, China.

Transgenic Research 10: 303–315, 2001.

Uniform GFP-expression in transgenic medaka (*Oryzias latipes*) at the F0 generation

Chi-Yuan Chou, Long-Shyan Horng & Huai-Jen Tsai*
Institute of Fisheries Science, National Taiwan University, Taipei, Taiwan, 106

Received 13 April 2000; revised 23 July 2000; accepted 4 December 2000

Key words: α-actin, β-actin, green fluorescent protein, inverted terminal repeats, mosaicism; transgenic fish

Abstract

A green fluorescent protein (GFP) cDNA flanked by inverted terminal repeats (ITR) of adeno-associated virus was constructed. The construct sharply improved the efficiency and specificity of the transient expression of genes driven by two general promoters (cytomegalovirus and medaka β-actin) and one muscle-specific promoter (zebrafish α-actin) in transgenic medaka. In addition, treatment with ITR sequence-containing constructs resulted in a dramatic increase in the number of embryos showing uniform GFP-expression at F0. Of the GFP-positive embryos, 34.6% (81/234), 10% (10/60), and 18% (38/212) showed homogenous GFP-expression for the derivative constructs of the cytomegalovirus, α-actin, and β-actin promoters, respectively. As a result of uniform GFP-expression, green fluorescence in founders was (a) extended for an entire lifetime without degradation, and (b) transmitted as a genetic trait to F1 and F2 progeny of some transgenic lines via Mendelian inheritance. A Southern blot analysis revealed a random integration of the transgene into the genome of founders and progeny in both head-to-tail and tail-to-tail concatemerization patterns. Interestingly, some transgenic medaka with uniform and strong fluorescence could be visually noticeable to the unaided eye.

Introduction

Due to its transparent chorion, easy injectability, controllable spawning, short generational lifespan, and ability to breed year-round, medaka (*Oryzias latipes*) is one of several vertebrates frequently used for *in vivo* studies of the functions, regulation, and inheritance of transgenes (Ozato et al., 1986). Transgenic fish studies make use of genes that (a) are driven by both heterologous and homologous sources of regulatory elements, and (b) originate from constitutive or tissue-specific expression genes. Control elements include genes from antifreeze protein (Fletcher et al., 1988; Du et al., 1992; Tsai et al., 1995a), mouse metallothionein (Maclean et al., 1987), chicken δ-crystalline (Ozato et al., 1986), carp β-actin (Hew, 1989; Iyengar & Maclean, 1995), salmon histone H3 (Chan & Devlin, 1993; Hanley et al., 1998), carp α-globin (Yoshizaki et al., 1991), *Xenopus* elongation factor (Lin et al., 1994), and a number of viruses (Guyomard et al., 1989; Zhang et al., 1990; Tsai et al., 1995b). However, there are important drawbacks to the use of these DNA elements in transgenic fish, including low expression efficiency (0–20%) (Culp et al., 1991; Higashijima et al., 1997) and the mosaic expression of transgene patterns (Stuart et al., 1990; Ju et al., 1999). At the F0 generation, transferred genes are rarely expressed as a homogeneous pattern; the uniform expression of a reporter gene in transgenic fish normally occurs in generation F1 or F2.

The microinjection into medaka eggs of a *lacZ* reporter gene driven by the medaka β-actin promoter results in the transient expression of the *lacZ* gene, even in the F1 generation, though expression is low and highly mosaic (Takagi et al., 1994). Hamada et al. (1998) reported a similar result in medaka embryos derived from eggs microinjected with green fluorescent protein (GFP) fused with the medaka β-actin promoter. Neither a heterologous nor homologous promoter serves to markedly decrease transgenic

* Author for correspondence: *E-mail:* hjtsai@ccms.ntu.edu.tw

mosaicism. Another approach to decreasing mosaic expression entails the introduction of foreign DNA fragments (i.e., sperm-mediated gene transfer) into the gametes of embryos prior to their first cleavage (Khoo et al., 1992; Symonds et al., 1994; Tsai et al., 1995a, 1997). However, it has been noted that the transferred DNA fragments are not evenly distributed throughout different tissue types of transgenic samples. Collas and Alestrom (1998) reported that the binding of nuclear localization signal (NLS) peptides to plasmid DNA increases gene transfer efficiency and enhances transient expression. Nevertheless, it is generally accepted that transgene integration mosaicism is inevitable (Liang et al., 2000). Recently Gibbs and Schmale (2000) reported that GFP driven by carp β-actin 5′ sequence, including insulator, promoter and first intron, was expressed from the egg to the adult. But this construct is less versatile in term of studying other promoters. Besides, the uniform phenotype is not stably inherited from the uniform GFP-expression parent. Therefore, the search continues for a simple but effective means of improving the F0 expression of transgene and the stable transmission of phenotype in transgenic fish.

Type 2 adeno-associated virus (AAV) contains an approximately 4.7 kb-long single-strand DNA genome with two inverted terminal repeats (ITR) (Srivastava et al., 1983); each ITR, consisting of 145 nucleotides, is in the form of a palindromic hairpin (Samulski et al., 1989). Fu et al. (1998) reported that the inclusion of an ITR in a DNA plasmid significantly increases the efficiency of transgene expression in *Xenopus* embryos. Accordingly, we set out to learn whether an ITR sequence can also enhance the ubiquitous and tissue-specific expression of transgenes in transgenic fish. If true, it might solve some of the difficulties encountered when using conventional DNA constructs in fish gene transfers. The present report describes the ability of a useful DNA construct, flanked at both ends by ITRs, to increase the efficient expression of transgenic genes in medaka. A uniform transgene expression was achieved in the F0 and the following two generations.

Materials and methods

Plasmids

All plasmids used in this study are presented in Figure 1. The pGREEN-LANTERN (pCMV-EGFP, 5.03 kb) (GIBCO contained an enhancer and promoter of an immediate early gene of cytomegalovirus (CMV) fused with mutated GFP cDNA (S65T) and SV40 polyA. EGFP cDNA (740 bp) was obtained from pEGFP-1 (Clontech) and digested with *Hin*dIII and *Not*I; ends were filled in. A blunted fragment was ligated to a *Stu*I fragment from vector CS2ITR (5 kb; provided by S.M. Evans) (Fu et al., 1998). The resulting plasmid, pCMV-EGFP-ITR (5.74 kb), contained a CMV promoter fused with EGFP cDNA, followed by SV40 polyA and flanked at both ends by AAV-ITR.

The pα-actin-EGFP (8 kb) (αp-G-BS; Higashijima et al., 1997) used in this study contained a zebrafish α-actin promoter fused with EGFP cDNA and SV40

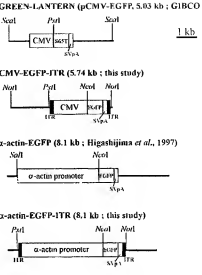

Figure 1. Plasmid constructs used for gene transfer. Thick lines represent plasmid vectors; scale bar indicates 1 kb. EGFP, hGFP, and S65T represent high expression mutations of GFP cDNA. CMV, cytomegalovirus immediate early enhancer/promoter; SvpA: polyadenylation signal of SV40; ITR: inverted terminal repeats of adeno-associated virus; SVin + pA: intron and polyadenylation signal of SV40 small t antigen gene; β-act-pA: polyadenylation signal of medaka β-actin gene.

polyadenylation signal (a gift from S.I. Higashijima). The zebrafish α-actin promoter was obtained from pα-actin-EGFP digested with *SalI* and *NcoI*. The resulting 3.9 kb fragment was ligated to a 4.2 kb *SalI-NcoI* fragment from pCMV-EGFP-ITR. This in turn resulted in an 8.1 kb pα-actin-EGFP-ITR plasmid, in which EGFP cDNA was driven by the α-actin promoter and flanked at both ends by AAV-ITR.

The pβ-actin-EGFP (10.6 kb) (pOBA-hGFP1; Hamada et al., 1998) used in this study contained a medaka β-actin promoter fused with hGFP1 cDNA, an intron of small t antigen, SV40 polyA, and polyA from the medaka β-actin gene (a gift from K. Ozato). The medaka β-actin promoter was obtained from pOBA-hGFP1 and digested with *SalI* and *NcoI*. The 3.8 kb fragment end-product was ligated to obtain a 4.2 kb *SalI-NcoI* fragment from pCMV-EGFP-ITR. The final result was an 8 kb pβ-actin-EGFP-ITR plasmid in which EGFP cDNA was driven by the β-actin promoter and flanked at both ends by AAV-ITR.

Preparation of microinjected DNA

All DNA plasmids were prepared via ultra-centrifugation with cesium chloride and ethidium bromide gradient (Radloff et al., 1967). Linearization of pCMV-EGFP and pCMV-EGFP-ITR was performed with *ScaI* and *NotI*, respectively. The molecular masses of the pCMV- EGFP and pCMV-EGFP-ITR fragments were 5.03 and 5.74 kb, respectively; for the pα-actin-EGFP-ITR and pβ-actin-EGFP-ITR DNA fragments, molecular masses were 5.1 and 5.0 kb, also respectively. The plasmids of pα- and pβ-actin-EGFP-ITR were restricted by *PstI* and *NotI*, pα-actin-EGFP and pβ-actin-EGFP were linearized by *SalI* and by *ApaI* and *SmaI*, respectively. The molecular mass for the pα-actin-EGFP DNA fragment was 8 kb and for the pβ-actin-EGFP DNA fragment 7.6 kb. All DNA fragments used for microinjection were eluted from agarose gel following electrophoresis.

Cytoplasmic microinjection

The procedures followed for cytoplasmic microinjection are described in detail in Kinoshita and Ozato (1995) and Kinoshita et al. (1996). Briefly, orange-red strain medaka were maintained under artificial conditions of 14 h light and 10 h darkness at 26°C and maintained on a diet of Tetramin (Tetra, Germany). Eggs were collected within 30 min of fertilization and attaching filaments removed. Fertilized eggs were kept at 6°C until microinjected with DNA fragments at a concentration of 10 μg/ml (300 pl) into their cytoplasm prior to the first cleavage. Injected eggs were incubated at 26°C in distilled water.

Fluorescent microscopy and cryosectioning

Embryos were observed under a bright field with a dissecting stereomicroscope (MZAPO, Leica, Germany). Dark field illumination for detecting green fluorescence was performed with a stereomicroscope equipped with a GFP Plus filter (480 nm). Photographs were taken using an MPS60 camera loaded with ISO 400 film and equipped with a controller for film exposure time (Leica, Germany).

In order to examine the distribution of GFP-expression in the tissues of transgenic medaka, 11 d post-fertilization larva which having uniform GFP-expression on appearance were sectioned and observed under fluorescent microscopy. Larva were fixed for 30 min in 4% paraformaldehyde at 4°C, embeded in cryomatrix (Shandon, USA) and frozen at −20°C. Cryostat sections (Cryostat Microtome, HM500 OM, Microm, Germany) with 15 μm thickness were mount on slides and observed the GFP fluorescence immediately.

Genomic DNA extraction

Genomic DNA was extracted using methods described in Chong and Vielkind (1989). Medaka samples were digested with proteinase K (100 μg/ml) solution containing 0.5% SDS for 16 h at 55°C. DNA was then purified using phenol-chloroform extraction and ethanol precipitation. Genomic DNA fragments extracted from embryos, fry and adult of medaka derived from β-actin-EGFP-ITR-injected eggs were digested with *NcoI* and *NotI*. Total restricted DNA was analyzed on a 0.8% agarose gel (FMC, U.S.A).

Southern blot analysis

Following electrophoresis, DNA was transferred onto a Hybond N^+ membrane (Amersham, U.S.A) and cross-linked with UV. Hybridization was carried out using a DIG- and isotope-labeled EGFP cDNA probe (740 bp). EGFP cDNA was prepared via PCR using a forward (5′-atggtgagcaagggcgaggag-3′) and a reverse primer (5′-gatctagagtcgcggccgct-3′). Amplification was performed for 35 cycles in a 50 μl

Table 1. Effects of various DNA constructs on survival and expression rates in transgenic medaka embryos

DNA construct			DNA fragment	Injected DNA size (kb)	No. of injected eggs	No. of surviving embryos (%)	No. of expression embryos (%)	No. of uniformity (%)		
Promoter	GFP	ITR						Stage 21 embryos (%)	Hatched fry (%)	Adult (%)
CMV	+	–	CMV-EGFP	5	226	184 (81.4)	179 (79.2)	0	0	0
CMV	+	+	CMV-EGFP-ITR	5.7	290	238 (82.1)	234 (80.7)	81 (27.9)	0	0
α-actin	+	–	α-actin-EGFP	8	51	46 (90.2)	43 (84.3)	0*	0	0
α-actin	+	+	α-actin-EGFP-ITR	5.1	79	66 (83.5)	60 (75.9)	6 (7.6)*	6 (7.6)	6 (7.6)
β-actin	+	–	β-actin-EGFP	7.6	124	109 (87.9)	109 (87.9)	0	0	0
β-actin	+	+	β-actin-EGFP-ITR	5	251	214 (85.3)	212 (84.5)	38 (15.1)	38 (15.1)	38 (15.1)
–	–	–	Control 1	–	102	94 (92.2)	0	0	0	0
–	–	–	Control 2	–	30	22 (73.3)	0	0	0	0
–	–	–	Control 3	–	56	40 (71.4)	0	0	0	0

Linearized DNA fragments (10 μg/ml) were microinjected into the cytoplasm of fertilized eggs at a volume of 300 pl. Surviving embryos at 36 h post-fertilization (stage 21) were examined to determine GFP expression. Uniformity indicated uniform transgenic GFP expression throughout the entire organism or in muscle tissue only. Controls consisted of parallel groups of non-CMV promoter-treated eggs (Control 1), non-α-actin promoter-treated eggs (Control 2), and non-β-actin promoter-treated eggs (Control 3). The percentages shown in parenthesis were calculated on the basis of the original numbers of injected eggs.
*GFP expressed exclusively in somites.

solution containing 2 pg of template DNA (pEGFP-1), 2.5 mM of each dNTP, 10 μM of each primer, 1X PCR buffer, and 2 units of *Taq* polymerase (GIBCO). For each cycle, denaturation was performed at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min (Perkin Elmer Centus). The resulting PCR product was labeled using a DIG or a radioisotope according to the manufacturer's protocol. Following the hybridization of the DIG-labeled probe, positive signals were visualized 2 h following the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim, Germany). Following the hybridization of the isotope-labeled probe, positive signals were visualized after sequentially washing and exposing to X-ray film at −70°C for several days.

Germ-line transmission

To determine the inheritance of the transferred DNA fragments, uniform GFP-expressions of F0 and F1 founders derived from β-actin-EGFP-ITR-injected embryos were crossed with wild varieties. The F1 and F2 progeny embryos were collected on day 1 and their green fluorescent signals detected at hatching (10 d following fertilization). Southern blot analysis was also performed on genomic DNAs extracted from F1 adults showing uniform and mosaic GFP-expression.

Results

Transgenic expression of GFP in CMV-EGFP- and CMV-EGFP-ITR-injected embryos

Linearized DNA fragments were injected into the cytoplasm of medaka embryos at the single-cell stage. While 79% of the CMV-EGFP-injected embryos tested GFP-positive (Table 1), GFP expression was mosaic and extremely faint (Figures 2A and 2B); none of the GFP-positive embryos showed uniform GFP-expression. In contrast, 80% of the CMV-EGFP-ITR-injected embryos tested GFP-positive, and approximately one-third of these showed GFP expression in almost every tissue at stage 21 (Table 1; Figures 2C and 2D). Neither CMV-EGFP- nor CMV-EGFP-ITR-injected embryos showed green fluorescence after stage 25 (52 h post-fertilization) (Figures 2E-2H).

Transgenic expression of GFP in α-actin-EGFP and α-actin-EGFP-ITR-injected embryos

In the α-actin-EGFP-injected embryos, GFP was primarily expressed in muscle tissue, especially in somites (Figures 3A and 3B). This localization of fluorescence was similar to that reported for the same fragment as expressed in zebrafish by Higashijima et al. (1997). Expression was sporadic at all embryonic stages without degradation in the fry stage. Mosaicism varied among the transgenic individuals. In addition,

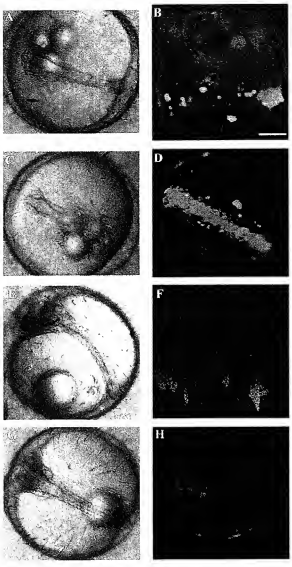

Figure 2. GFP expression in medaka embryos injected with GFP cDNA driven by a CMV promoter. Green fluorescence was observed in CMV-EGFP-injected (A, B, E, F) and CMV-EGFP-ITR-injected medaka embryos (C, D, G, H) at stages 21 (32 h post-fertilization for A, B, C and D) and 25 (52 h post-fertilization for E, F, G and H), respectively. Left panels were exposed under light field illumination; right panels were under dark field illumination. At stage 25, GFP expression decreased dramatically in both CMV-EGFP- and CMV-EGFP-ITR-injected embryos. White scale bar indicates 0.2 mm.

GFP was expressed from stage 17 (early neurula stage) onwards in α-actin-EGFP-injected embryos.

In the α-actin-EGFP-ITR-injected embryos, GFP was expressed exclusively in muscle tissue. In most of these embryos, over 50% of somites tested GFP-positive after stage 30 (35 somite stage). Some GFP signals were observed at stage 21 (6 somite stage), but widespread observation only occurred after stage 30 (Figure 3C). In total, approximately 10% of the GFP-positive embryos exhibited uniform transgenic GFP expression throughout their somites (Table 1 and Figure 3D). These uniform GFP-expression embryos produced green fluorescence throughout their lives, that was, for at least one year.

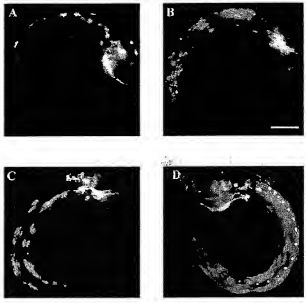

Figure 3. Expression of transgenic GFP driven by the α-actin promoter in medaka embryos at stages 31 (4 d post-fertilization; A, B and C) and 33 (4.5 days post-fertilization; D), respectively. A and B: α-actin-EGFP-injected embryos; GFP was sporadically expressed, primarily in somites. C: α-actin-EGFP-ITR-injected embryos: GFP was tissue-specific and mosaically expressed in muscle. D: α-actin-EGFP-ITR-injected embryos: GFP was uniformly expressed in muscle. White bar indicates 0.2 mm.

Transient expression of GFP in β-actin-EGFP- and β-actin-EGFP-ITR-injected embryos

In the β-actin-EGFP-injected embryos, GFP was primarily expressed in the epidermis, blood vessels, muscle tissue, yolk sac, notochord, finray, and eyes (Figures 4C and 4E); GFP expression also appeared in the hearts and circulating blood cells of some embryos (data not shown). Like the GFP expression in α-actin-EGFP-injected embryos, GFP expression in β-actin-EGFP-injected embryos occurred sporadically at all embryonic stages, and some signals lasted into the fry stage. As with α-actin-EGFP-ITR-injected embryos, β-actin-EGFP-ITR injected embryos exhibited greater GFP expression than those embryos injected with the plasmid construct without ITR (Figure 4D). Over 50% of external body of most of the β-actin-EGFP-ITR-injected embryos appeared GFP-expression. Approximately 18% of the GFP-positive embryos (15% of total injected embryos) showed uniform expression (Table 1; Figures 4A, 4B, and 4F). Green fluorescence appeared uniformly in every tissue of transgenic larvae when cryosections were performed and observed under fluorescent microscopy (Figure 4G). This GFP-expression maintained for two generations, that is, to at least F2 progeny. The green fluorescence in some transgenic embryos was strong enough to be visible to the unaided eye.

Overall, no differences were observed in the frequencies of abnormal transgenic embryos injected with CMV-, α-actin-, or β-actin-derived DNA fragments with or without ITR sequences (Table 1). Moreover, even when CMV-EGFP-, α-actin-EGFP-, or β-actin-EGFP-DNA fragments were injected at ten-fold concentrations (100 μg/ml), transgenic GFP expression did not reach levels where green fluorescence signals were produced by CMV-EGFP-ITR-, α-actin-EGFP-ITR-, or β-actin-EGFP-ITR-injected embryos; higher concentrations instead resulted in greater mortality.

Southern blot analysis of genomic DNA of F0 derived from β-actin-EGFP-ITR-injected embryos

At stages 29 (3 d post-fertilization) and 31 (4 d post-fertilization), genomic DNAs from GFP-positive F0 embryos derived from β-actin-EGFP-ITR-injected eggs were extracted, digested with *Nco*I and *Not*I, and analyzed by Southern blot hybridization using a DIG-labeled probe. Concatemerizations of the transferred DNA fragments that are theoretically possible

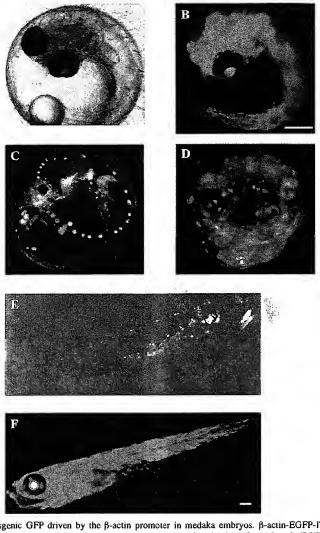

Figure 4. Expression of transgenic GFP driven by the β-actin promoter in medaka embryos. β-actin-EGFP-ITR-injected embryos at stage 36 (6.5 d post-fertilization) were exposed under light (A) and dark (B) field illumination. Other β-actin-EGFP-ITR-injected embryos were exposed under dark field illumination at stage 38 (8 d post-fertilization) (D) and at hatching (12 d post-fertilization) (F). β-actin-EGFP-injected embryos were exposed under dark field illumination at stage 38 (C) and at hatching (E). (G). Cryosection of 11-d larvae derived from β-actin-EGFP-ITR-injected embryo. Scale bar indicates 0.2 mm. Film-exposure time for figures (B) and (F) was 1.0 s, for Figure (D) 4.2 s, for Figure (G) 3 min and 41 s, and for the remaining Figures 8 s.

occurred are illustrated in Figure 5A. Southern blot analyses showed that positive bands with molecular masses of 4.5, 4.7, 5.2, and 4.7 kb in some transgenic medaka whose GFP was expressed mosaically (lanes 1–4 in Figure 5B). Two positive bands (4.5 and 23.1 kb) were also observed in a single medaka showing mosaic GFP expression (lane 8 in Figure 5B), as well as two positive bands (2.2 and 5.2 kb) in transgenic medaka showing uniform GFP expression (lanes 5–7 in Figure 5B). As expected, no positive bands were observed in medaka from the non-transgenic control group (lane N in Figure 5B).

Germ-line transmission of foreign GFP gene

Transgenic medaka derived from embryos microinjected with β-actin-EGFP-ITR fragments and uniformly expressing the foreign GFP gene were crossed with a

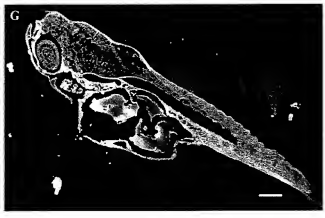

Figure 4. (*continued*)

wild-type medaka strain. Results showed that F1 progeny from three of the nine lines (lines 1, 4, and 6) had the same uniform GFP expression as did their F0 parent. Of the F1 embryos derived from line 4 (male), 46.8% (182/389) showed uniform GFP expression. When three F1 female progeny from line 4 (4F1-1, -2, and -3) were crossed with wild-type medaka individuals, 56.5% (65/115), 51.2% (21/41), and 46.2% (18/39) of the embryos of their F2 progeny showed uniform GFP expression, respectively. These percentages were close to what would be expected from the Mendelian inheritance of green fluorescent signals in F1 and F2 generations. However, one of the nine lines (line 2) showed a mosaic pattern; the remaining lines were sterile.

Genomic DNA from F1 progeny showing uniform GFP expression (lines 1 and 4) and that showing mosaic GFP expression (line 2) were extracted, digested with *Nco*I and *Not*I, and subjected to Southern blot hybridization using a DIG-labeled probe. Results revealed two positive bands with molecular masses of 2.2 and 5.2 kb (data not shown), which were as same as those of their transgenic F0 generation parents. However, when a radioisotope-labeled probe was used, in addition that two prominent bands of 2.2 and 5.2 kb were shown, several extra faint bands with molecular masses of 7.9, 3.8, 1.7 and 1.1 kb were noted for line 1; 9.5 and 3.1 kb were noted for line 2; and 8.9 and 1.1 kb were noted for line 4 (Figure 5C).

In summary, both, what appears to be, head-to-head and tail-to-tail transgene arrangements were detected, particularly in those fish displaying uniform GFP-expression in both F0 and F1 generations.

Discussion

When a DNA fragment consisting of a reporter or target gene of homologous or heterologous origin is transferred, it is very common to find mosaic, transient, or variegated expressions of the gene in transgenic fish. As Liang et al. (2000) point out, mosaic transgene integration in the germ lines of F0 founders is frequently detected even when NLS peptide-DNA complexes are employed. In other words, homogeneous expression of a transferred gene in the F0 generation is a rare occurrence, unlike expression in the F1 generation. Although Gibbs and Schmale (2000) demonstrated that transgenic GFP could be expressed throughout the life, the phenotypic difference occurred between the transgenic lines. In addition, the construct they used was not convenient to be applied in other desired promoter. Here, we believe the results of this study show that ITR technology can be used to overcome these disadvantages.

Constructing plasmids for transferring into medaka embryos, we flanked both ends of the GFP cDNA reporter gene with AAV-ITR. Three promoters from different gene sources were incorporated into the design: an immediate early gene of CMV, the muscle-specific α-actin gene of zebrafish, and the ubiquitous β-actin gene of medaka. The data indicate a remarkable improvement in the transient expression of those promoters in transgenic fish due to the incorporation of the ITR sequence. It is expected that the biological characteristics of this special DNA construct will function in transgenic medaka in a manner comparable to that reported by Fu et al. (1998) in *X. laevis* embryos.

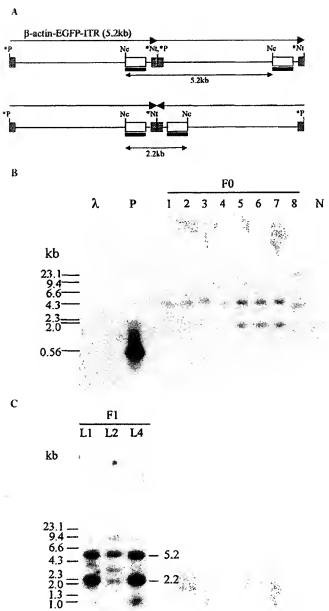

Figure 5. Southern blot analyses of genomic DNAs isolated from embryos, fry and adult medaka derived from β-actin-EGFP-ITR-injected eggs. A: schematic diagram of *PstI-NotI* linearized β-actin-EGFP-ITR construct containing GFP cDNA (open box) and flanked by ITR (hatched box). The products below (5.2 and 2.2 kb) were expected for head-to-tail and tail-to-tail concatemerization, respectively. Probes were indicated by thick blacken lines. Nc: *NcoI*; Nt: *NotI*; P: *PstI*. *: the restriction site was modified during concatemerization. B: Southern blot analysis of genomic DNA extracted from F0 founders of GFP-positive transgenic medaka and digested with *NcoI* and *NotI*. Lane λ *HindIII*-cut λ genome; lane P: positive control (1 ng EGFP cDNA [740-bp]); lanes 1–4: embryos with sporadic GFP-expression at 3 d post-fertilization; lanes 5–7: embryos with uniform GFP-expression at 4 d post-fertilization; lane 8: a single embryo with sporadic GFP-expression at 4 d post-fertilization; lane N: negative control, wild-type embryo at 4 d post-fertilization. C: Southern blot analysis of genomic DNA extracted from F1 progeny of GFP-positive transgenic lines 1 (L1 uniform GFP-expression), 2 (L2 mosaical GFP-expression) and 4 (L4 uniform GFP-expression), and digested with *NcoI* and *NotI*.

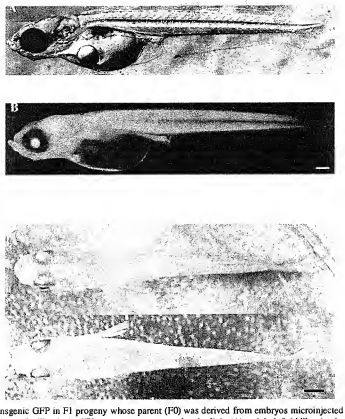

Figure 6. Expression of transgenic GFP in F1 progeny whose parent (F0) was derived from embryos microinjected with a β-actin-EGFP-ITR fragment. Hatching fry (12 d post-fertilization) of F1 progeny were exposed under light (A) and dark field illumination (B). (C), adult wild strain (upper) and adult F1 progeny with green fluorescence (lower) photographed under natural lighting. White scale bar in (B) indicates 0.2 mm; black one in (C) indicates 2 mm. Film-exposure time for (B) and (C) were 1.0 and 0.01 s, respectively.

Due to its location in the nuclear membrane (Weitzman et al., 1996), the AAV-ITR sequence makes the embryonic distribution of the transferred DNA fragment more even. As a result, fragment expression is more efficient and tissue-specific.

The observed temporal activation of the ITR-flanked muscle α-actin promoter agrees with the activation timing of the endogenous gene at stage 21 and maximal expression at stage 30, an expression stage similar to that reported in mice (Sassoon et al., 1988). By contrast, the activation of the non-ITR muscle α-actin promoter occurred at stage 17, earlier than expected in normal development. We, therefore, suggest that the addition of AAV-ITR not only enhances tissue-specificity, but also enhances the expression of exogenously introduced genes under the control of a specific promoter.

In addition, regardless of promoter type, none of the transgenic embryos injected with non-ITR-containing DNA fragments showed uniform GFP expression at F0; injection with ITR-containing constructs raised the percentage of uniform GFP-expression embryos at F0 to between 10 and 18%. The number of embryos showing uniform and persistent GFP-expression in the β-actin-injected group was higher than that in the α-actin-injected group, a difference that may be attributed to the medaka-origin of the β-actin promoter. Transgenic medaka injected with both α- and β-actin-EGFP-ITR fragments retained their green fluorescence without degradation throughout their lives; this trait of homogeneous GFP expression was inherited by F1 and F2 progeny (Figures 6A and 6B). To our knowledge, this is the first report of a transgenic GFP reporter gene being uni-

formly and strongly expressed in the F0 generation and being possible to identify transgenic medaka with the unaided eye (Figure 6C).

Unlike the persistent GFP expression observed in embryos injected with the α- and β-actin-promoters, embryos injected with the CMV promoter failed to show transient GFP-expression in transgenic medaka after stage 25 (52 h post-fertilization). This finding is similar to Tsai et al.'s (1995b) report of undetected *lacZ* expression in CMV-*lacZ*-injected medaka embryos at 48 h post-fertilization. A probable explanation is that the epigenetic modification of the CMV promoter is due to the endogenous methylation of cytosine in CpG dinucleotides, which results in suppressing transgene expression (Kass et al., 1997; Collas, 1998). On the other hand, embryos injected with the α- and β-actin-promoters, both of fish origin (Higashijima et al., 1997; Hamada et al., 1998), showed prolonged GFP expression in transgenic medaka.

Unexpectedly, some F0 lines failed to transmit uniform GFP expression to their progeny due to their mosaic GFP expression patterns. Xiao et al. (1996) and Wu et al. (1998) both reported a high-level, persistent transgene expression of recombinant AAV (rAAV) in muscle cells, neurons, and non-dividing cells, and Duan et al. (1998) found that the episomal persistence of rAAV was correlated with its long-term transgenic expression. The false-positive result of uniform GFP expression in founders may be explained by the transgenic GFP gene being strongly expressed in skin and muscle tissue, but intermittently expressed in other tissue types, resulting in mosaic germ-line transmission. Another possibility is that the transferred AAV-ITR-flanked gene may be lost, or its expression may be turned off, during gamete formation. This explanation has support from Zhang and Fuleihan (1999), who reported the loss of some rAAV DNA and the silencing of transgenic expression in some cell lines.

We found that female trangenic medaka were capable of transmitting GFP mRNA to such a degree that newly fertilized eggs projected a strong green fluorescence. However, the intensity of the signal decreased during embryonic development, and disappeared entirely at the hatching stage (8–10 d post-fertilization). No positive bands were observed during PCR or Southern blot searches for transgenic GFP fragments in the transiently green eggs (data not shown). Therefore, green fluorescence in hatching eggs was used to study the Mendelian inheritance of the transgene. Approximately 50% of line 4 F1 and F2 progeny tested GFP-positive; this percentage is close to what one would expect from the Mendelian inheritance of a transgene from a uniformly GFP-expressed F0 founder. This uniformly green phenotype was transmitted to F2 progeny to such a strong degree that it was easily noticeable.

Naturally occurring AAV has been reported as showing site-specific integration characteristics at chromosome 19 in the human genome (Samulski et al., 1991; Kotin et al., 1992). On the other hand, rAAV persistence has been attributed to both episomal (Flotte et al., 1994) and random (Kearns et al., 1996; Ponnazhagan et al., 1997) integration in human cell lines. In the present study, Southern blot analysis demonstrated random rAAV integration into the medaka genome at a single locus. The transfer of foreign DNA fragments containing the AAV-ITR sequence strongly enhanced the transient expression of GFP. This effect makes medaka a more practical animal subject for transgene studies. Its transparent physical characteristic makes it easy to note strong GFP expression, which is helpful in screening for the uniform expression of other transgenes.

Acknowledgements

The authors wish to thank Drs. S.M. Evans, S.I. Higashijima, and K. Ozato for their generous gifts of plasmids. We also thank Prof. Jui-Ping Lin, Department of Zoology, National Taiwan University, for providing Cryostat Microtome and instructing cryosection. This was supported by the grant from National Science Council, Republic of China, NSC 88-2311-B002-034-B24.

References

Chan WK and Devlin RH (1993) Polymerase chain reaction amplification and functional characterization of sockeye salmon histone H3, metallothionein-B and protamine promoters. *Mol Marine Biol Biotechnol* 1: 97–105.

Chong SSC and Vielkind JR (1989) Expression and fate of CAT reporter gene microinjected into fertilized medaka (*Oryzias latipes*) eggs in the form of plasmid DNA, recombinant phage particles and its DNA. *Theor Appl Genet* 78: 369–380.

Collas P (1998) Modulation of plasmid DNA methylation and expression in zebrafish embryos. *Nucleic Acids Res* 26: 4454–4461.

Collas P and Alestrom P (1998) Nuclear localization signals enhance germline transmission of a transgene in zebrafish. *Transgenic Res* 7: 303–309.

Culp P, Nusslein, Volhard C and Hopkins N (1991) High-efficiency germ-line transmission of plasmid DNA sequences injected into

fertilized zebrafish eggs. *Proc Natl Acod Sci USA* **18**: 7953–7957.

Du SJ, Gong Z, Fletcher GL, Shears MA and Hew CL (1992) Growth hormone gene transfer in Atlantic salmon: Use of fish antifreeze/growth hormone chimeric gene constructs. In: Hew CL and Fletcher GL (eds), *Transgenic Fish* (pp. 176–189) World Scientific Publishing, Singapore.

Duan D, Sharma P, Yang J, Yue Y, Dudus L, Zhang Y, Fisher KJ and Engelhardt JF (1998) Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J Virol* 72: 8568–8577.

Fletcher GL, Shears MA, King MJ, Davies PL and Hew CL (1988) Evidence of antifreeze protein gene transfer in Atlantic salmon (*Salmo salar*). *Can J Fish Aquo Sci* **45**: 352–357.

Flotte TR, Afione SA and Zeitlin PL (1994) Adeno-associated virus vector gene expression occurs in nondividing cells in the absence of vector DNA integration. *Am J Respir Cell Mol Biol* **11**: 517–521.

Fu Y, Wang Y and Evans SM (1998) Viral sequences enable efficient and tissue-specific expression of transgenes in *Xenopus*. *Nat Biotechnol* **16**: 253–257.

Gibbs PDL and Schmale MC (2000) GFP as a genetic marker scorable throughout the life cycle of transgenic zebrafish. *Marine Biotechnol* 2: 107–125.

Guyomard R, Chourrout D and Houdebine LM (1989) Production of stable transgenic fish by cytoplasmic injection of purified genes. *Symp Mol Cellular Biol* **87**: 9–18.

Hamada K, Tamaki K, Sasado T, Watai Y, Kani S, Wakamatsu Y, Ozato K, Kinoshita M, Kohno R, Takagi S and Kimura M (1998) Usefulness of the medaka β-actin promoter investigated using a mutant GFP reporter gene in transgenic medaka (*Oryzias latipes*). *Mol Marine Biol Biotechnol* 7: 173–180.

Hanley S, Smith TJ, Muller F, Maclean N, Uzbekova S, Prunet P and Breton B (1998) Isolation and functional analysis of the histone H3 promoter from Atlantic salmon (*Salmo solar* L.). *Mol Marine Biol Biotechnol* 7: 165–172.

Hew CL (1989) Transgenic fish: Present status and future directions. *Fish Physiol Biochem* **7**: 409–413.

Higashijima SI, Okamoto H, Ueno N, Hotta Y and Eguchi G (1997) High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. *Dev Biol* **192**: 289–299.

Iyengar A and Maclean N (1995) Transgene concatemerisation and expression in rainbow trout (*Oncorhynchus mykiss*). *Mol Marine Biol Biotechnol* **4**: 248–254.

Ju B, Xu Y, He J, Liao J, Yan T, Hew CL, Lam TJ and Gong Z (1999) Faithful expression of green fluorescent protein (GFP) in transgenic zebrafish embryos under control of zebrafish gene promoters. *Dev Genet* **25**: 158–167.

Kass S, Pruss D and Wolffe A (1997) How does DNA methylation repress transcription? *Trends Genet* **13**: 444–449.

Kearns WG, Afione SA, Fulmer SB, Pang MC, Erikson D, Egan M, Landrum MJ, Flotte, TR and Cutting GR (1996) Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line. *Gene Ther* 3: 748–755.

Khoo HW, Ang LH and Wong KY (1992) Sperm cells as vectors for introducing foreign DNA into zebrafish. *Aquoculture* **107**: 1–19.

Kinoshita M and Ozato K (1995) Cytoplasmic microinjection of DNA into fertilized medaka (*Oryzios latipes*) eggs. *Fish Biol J MEDAKA* 7: 59–64.

Kinoshita M, Toyohara H, Sakaguchi M, Inoue K, Yamashita S, Satake M, Wakamatsu Y and Ozato K (1996) A stable line of transgenic medaka (*Oryzias latipes*) carrying the CAT gene. *Aquaculture* **143**: 267–276.

Kotin RM, Linden RM and Berns KI (1992) Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. *EMBO J* **11**: 5071–5078.

Liang MR, Alestrom P and Collas P (2000) Glowing zebrafish: Integration, transmission, and expression of a single luciferase transgene promoted by noncovalent DNA-nuclear transport peptide complexes. *Mol Reprod Dev* **55**: 8–13.

Lin S, Yang S and Hopkins N (1994) *lacZ* expression in germ-line transgenic zebrafish can be detected in living embryos. *Dev Biol* **161**: 77–83.

Maclean N, Penman D and Zhu Z (1987) Introduction of novel genes into fish. *Biotechnology* **5**: 257–261.

Ozato K, Kondoh H, Inohara H, Iwamatsu T, Wakamatsu Y and Okada TS (1986) Production of transgenic fish: Introduction and expression of chicken δ-crystallin gene in medaka embryos. *Cell Differ* **19**: 237–244.

Ponnazhagan S, Erikson D, Kearns WG, Zhou SZ, Nahreini P, Wang XS and Srivastava A (1997) Lack of site-specific integration of the recombinant adeno-associated virus 2 genomes in human cells. *Hum Gene Ther* **8**: 275–284.

Radloff R, Bauer W and Vinograd J (1967) A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: The closed circular DNA in HeLa cells. *Proc Natl Acad Sci USA* **57**: 1514–1521.

Samulski RJ, Chang L-S and Shenk T (1989) Helper-free stocks of adeno-associated viruses: Normal integration does not require viral gene expression. *J Virol* **63**: 3822–3828.

Samulski RJ, Zhu X, Xiao X, Brook JD, Housman DE, Epstein N and Hunter LA (1991) Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J* **10**: 3941–3950.

Sassoon DA, Garner I and Buckingham M (1988) Transcripts of alpha-cardiac and alpha-skeletal actins are early markers for myogenesis in the mouse embryo. *Development* **104**: 155–164.

Srivastava A, Lusby EW and Berns KI (1983) Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J Virol* **45**: 555–562.

Stuart GW, Vielkind JR, McMurray JV and Westerfield M (1990) Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. *Development* **109**: 577–584.

Symonds JE, Walker SP, Sin FYT and Sin I (1994) Development of a mass gene transfer method in chinook salmon: Optimization of gene transfer by electroporated sperm. *Mol Marine Biol Biotechnol* 3: 104–111.

Takagi A, Sasado T, Tamiya G, Ozato K, Wakamatsu Y, Takeshita A and Kimura M (1994) An efficient expression vector for transgenic medaka construction. *Mol Marine Biol Biotechnol* 3: 192–199.

Tsai HJ, Tseng FS and Liao I (1995a) Electroporation of sperm to introduce foreign DNA into the genome of loach (*Misgurnus anguillicoudatus*). *Can J Fish Aquat Sci* **52**: 776–778.

Tsai HJ, Wang SH, Inoue K, Takagi S, Kimura M, Wakamatsu Y and Ozato K (1995b) Initiation of the transgenic *lacZ* gene expression in medaka (*Oryzios latipes*) embryos. *Mol Marine Biol Biotechnol* 4: 1–9.

Tsai HJ, Lai CH and Yang HS (1997) Sperm as a carrier to introduce an exogenous DNA fragment into the oocytes of Japanese abalone (*Haliotis diversicolor supertexta*). *Transgenic Res* 6: 85–95.

Weitzman M.D, Fisher KJ and Wilson JM (1996) Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication center. *J Virol* **70**: 1845–1854.

Wu P, Phillips MI, Bui J and Terwilliger EF (1998) Adeno-associated virus vector-mediated transgene integration into neurons and other nondividing cell targets. *J Virol* **72**: 5919–5926.

Xiao X, Li J and Samulski RJ (1996) Efficient long-term gene transfer into musele tissue of immunocompetent mice by adeno-associated virus vector. *J Virol* **70**: 8098–8108.

Yoshizaki G, Oshiro T and Takashima F (1991) Introduction of carp α-globin into rainbow trout. *Nippon Suisan Gakkaishi* **57**: 819–824.

Zhang P, Hayat M, Joyce C, Gonzalez-Villasenor LI, Lin CM, Dunham RA, Chen TT and Powers DA (1990) Gene transfer, expression and inheritance of pRSV-rainbow trout-GH cDNA in the common carp, *Cyprinus carpio*. *Mol Reprod Dev* **25**: 13–25.

Zhang PX and Fuleihan RL (1999) Transfer of activation-dependent gene expression into T-cell lines by recombinant adeno-associated virus. *Gene Ther* **6**: 182–189.

FISHERIES SCIENCE 2004; 70: 645–649

Transgenic medaka with brilliant fluorescence in skeletal muscle under normal light

MASATO KINOSHITA*

Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

ABSTRACT: *Green fluorescent protein* (*GFP*) and *red fluorescent protein* (*RFP*) genes regulated by the medaka skeletal muscle actin promoter were microinjected into fertilized d-rR medaka eggs to establish transgenic medaka lines. Intense fluorescence was detected in skeletal muscle. During development, GFP and RFP became detectable in anterior somites at the 12- and 30-somete stages, respectively. After hatching, intense fluorescence in skeletal muscle enabled individual fish to be identified under normal lighting without fluorescent microscopy. Fluorescence was also observed in the gills and esophagus of the adult fish. These data indicated that medaka lines are convenient not only for the study of skeletal muscle but also for the identification of cells or individuals in various studies.

KEY WORDS: **green fluorescent protein, medaka, red fluorescent protein, skeletal muscle actin, transgenesis.**

INTRODUCTION

Medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) have features that allow them to be useful models.[1,2] In addition to natural qualities, artificially additional advantages have elevated their significance. One such advantage is that organs can be labeled. Proteins such as green fluorescent protein (GFP) and red fluorescent protein (RFP) are popular label substances because they are detectable in living organisms.[3] Some transgenic medaka strains with GFP-labeled tissue have been established, such as those with green fluorescence, in almost all tissues without skeletal muscle,[4] in germ cells,[5] in mainly skeletal muscle and eyes,[6,7] and in all cells.[8] Some GFP-labeled transgenic zebrafish strains have also been established, including green fluorescence in the whole body,[9,10] in cranial motor neurons[11] and in skeletal muscle.[9,12,13]

One goal of the present transgenic study was to produce transgenic medaka strains labeled with tissue-specific fluorescent proteins. Transgenic medaka strains harboring fluorescent protein genes regulated by the regulatory region of the medaka skeletal muscle actin gene were indeed established. These fish were so intensely fluorescent that transgenic individuals were easily identified by the color of the body. The use of these strains in many types of studies is discussed.

MATERIALS AND METHODS

The plasmid *pOlMA1-GFP* expressing green fluorescent protein (GFP) expression was a gift from Dr Takehiro Kusakabe. The plasmid, designated as MA1(– 1430)intron(+) in the original paper, contains the *GFP* gene regulated by the medaka skeletal muscle actin promoter and enhancer.[14] The expression plasmid, *pOlMA1-DsRed2*, expressing red fluorescent protein (RFP) was constructed by inserting an *Eco*RI/*Sal*I fragment containing the medaka skeletal muscle actin promoter and enhancer in *pOlMA1-GFP* into the *Eco*RI/*Sal*I restriction sites of *pDsRed2-1* vector (Clontech, Palo Alto, CA, USA). These plasmids were injected into fertilized eggs of the d-rR strain before the first cleavage as described by Kinoshita *et al.*[4] with slight modifications as follows. Plasmids were dissolved in 50 ng/mL Yamamoto's solution (0.75% NaCl, 0.02% KCl, 0.02% $CaCl_2$, and 0.002% $NaHCO_3$, pH 7.3).[15] Eggs were stored at 4°C in distilled water until microinjection. Transgenic medaka lines were established as described.[4] Transgenes were

*Corresponding author: 81-75-753-6213.
Fax: 81-75-753-6223. Email: kinoshit@kais.kyoto-u.ac.jp

The transgenic medaka strains described here are available for research use through the 'Bioresource Project'. Please contact Professor Hiroshi Mitani (mitani@mail.k.u-tokyo.ac.jp).

Received 25 November 2003. Accepted 6 April 2004.

detected by polymerase chain reaction (PCR) under the following conditions: 30 s at 96°C, 30 s at 60°C, and 45 s at 72°C for 30 cycles. The PCR primers were 5'- GACATTTGTGCTGTCCAGCTCACAC-3' and 5'- GACTGCGGCCGCTTACTTGTACAGCTCGTCCAT-3' (to detect *OlMA1-GFP*) or 5'- CGATGGATCCGTCGCGGCCGCTACAGGAACA-3' (to detect *OlMA1-DsRed2*). Green and red fluorescence in embryonic or adult tissues was detected using a fluorescence stereo-microscope (Leica FL III; Leica, Nussloch, Germany) and GFP2 or TXR filter sets, respectively. Chorion was sometimes partially removed by digestion with pronase (Roche, Mannheim, Germany).

RESULTS

Establishment of transgenic medaka strains

Of 375 fertilized eggs injected *pOlMA1-GFP*, 28 embryos reached adulthood and one female harbored the transgene in germ cells. Of 20 fertilized eggs injected with *pOlMA1-DsRed2*, 10 embryos reached adulthood and one male harbored the transgene in germ cells. A transgenic F_1 fish harboring *pOlMA1-GFP* or *pOlMA1-DsRed2* was mated with non-transgenic medaka to establish transgenic strains as described.[4] The frequencies of transgenics in the F_1 generation were 0.9% (4 of 443 individuals) and 7.7% (18 of 233) for *OlMA1-GFP* and *OlMA1-DsRed2* transgenics, respectively, indicating mosaic integration in F0 germ cells. On the contrary, the rates of Mendelian inheritance in the F_2 generation were 51% (54 of 106) and 48% (59 of 122) for *OlMA1-GFP* and *OlMA1-DsRed2* transgenics, respectively, suggesting that both *OlMA1-GFP* and *OlMA1-DsRed2* were integrated into one or a very close locus within a chromosome. This integration profile was also supported by the fact that the segregation ratio of GFP negative and DsRed2 negative : GFP positive and DsRed2 negative : GFP negative and DsRed2 positive : GFP positive and DsRed2 positive in the F_3 generation derived from an F_2 male heterozygous for the *GFP* gene and an F_2 female heterozygous for the *DsRed2* gene was about 1 : 1 : 1 : 1 (40, 38, 42, and 47 of 167 tested individuals).

Expression profile of GFP or RFP

Faint GFP fluorescence was observed in the embryonic body and yolk sac in an early embryo of the *OlMA1-GFP* transgenic strain derived from a transgenic female, but the fluorescence disappeared by the hatching stage (data not show).

Figure 1 shows the expression profile observed in F_2 individuals. Green fluorescence initially appeared only in anterior somites at the 12-somite stage (stage 23; Fig. 1a–c). A few heart cells of some embryos contained GFP fluorescence, but the fluorescence disappeared by hatching. In contrast, red fluorescence appeared at the 30-somite stage (stage 28). The delayed appearance of red fluorescence is due to the fact that the chromophore maturation of DsRed is slower than that of GFP,[16,17] However, the expression of DsRed2 was consistent with that of GFP throughout the lifetime of the fish. Therefore, mainly GFP expression profile is now shown. This expression profile was consistent with that of the skeletal muscle actin gene (*OlMA1*) described by Kusakabe *et al.*[14] indicating that the expression profiles of GFP and DsRed2 paralleled that of OlMA1.

Figure 2 shows the GFP expression profile in hatchlings. Fluorescence was obvious in skeletal muscle of the trunk (Fig. 2a,b) and sufficiently intense to distinguish individuals under daylight or fluorescent light with the naked eye (Fig. 2c). Fluorescence was also emitted from the skeletal muscle of the jaw and fin bud (Fig. 2c–e). In addition to these, green stripes that stretched from the heart to the air bladder appeared on the abdominal surface, suggesting the presence of muscle fiber that might regulate yolk sac contraction (Fig. 2f–h). This finding highlighted one advantage of the transgenic strain. The expression profile of the brilliant fluorescence was obvious, so the expression profile of α-actin was easy to follow in detail.

Fluorescence in the trunk skeletal muscle of adults was extremely intense and the body color of each fish appeared green or red to the naked eye under daylight or fluorescent light (Fig. 3a). The fluorescence of GFP was also obvious in the gills and esophagus (Fig. 3c–h). Some fish did not express GFP in heart (for example, arrowhead in Fig. 3c–e), but GFP fluorescence was evident in the ventricle of others and the intensity and area varied among individual fishes (Fig. 3i–k). The reason why expression differs among individuals is unclear. Fluorescence was not observed in any other tissues. In addition to green and red fluorescence, medaka labeled with orange fluorescence (intermediate between green and red) were produced from parents labeled with green and red fluorescence (Fig. 3a). Orange, as well as green and red fluorescence was discernible with the naked eye under both daylight and fluorescent light.

DISCUSSION

Some investigators have produced transgenic fish with muscles that express fluorescence. Higash-

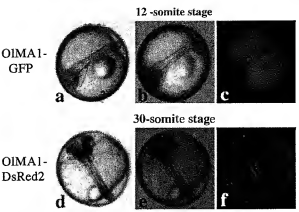

Fig. 1 Expression of fluorescence in transgenic F_2 embryo. F_2 embryo of transgenic medaka strain harboring *green fluorescent protein (GFP)* gene or *DsRed2* gene regulated by *medaka skeletal muscle actin gene*. (a–c) GFP expression was detectable from the 12-somite stage and (d–f) red fluorescent protein (RFP) expression from the 30-somite stage. (a,d) Bright field images. (b,e) Fused images of (a,c) or (d,f) respectively. (c,f) Fluorescent images.

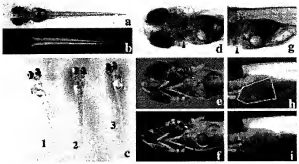

Fig. 2 Expression of fluorescence in transgenic F_2 hatchling. Intense green fluorescence in trunk muscle of hatchling harboring *OlMA1-GFP* (a,b are dorsal view of hatchling). Fluorescence of trunk muscle is intense enough to identify hatchlings by body color (1, 2 and 3 in c are hatchlings of non-transgenics, harboring *OlMA1-DsRed2*, and *OlMA1-GFP*, respectively). Image in panel c was taken under fluorescent light without fluorescent microscope. (d–f) Ventral views of jaw and abdomen of hatchling harboring *OlMA1-GFP*. Fluorescence in muscle of jaw and fin bud, but not in heart (arrowheads in d and g). (g–i) Lateral views of hatchling harboring *OlMA1-GFP*. Strip of green fluorescence extends from heart to air bladder (boxed by white line in h). (a,c,d,g) Bright field images. (b,f,i) Fluorescent images. (e,h) Fused images of (d,f) or (g,i) respectively.

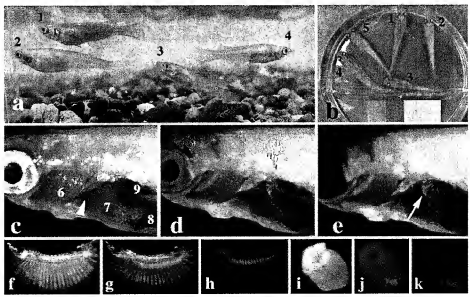

Fig. 3 Expression of fluorescence in adult and juvenile transgenic fish. (a) Lateral views of adult fish under fluorescent light without fluorescent microscope. (b) Dorsal view of juvenile fish under daylight without fluorescent microscope. (c–e) Ventral views of internal organs. No fluorescence was observed in the heart of this individual (arrowhead in c). Green fluorescence was observed in gill (6 in c) and esophagus (arrow in e). (f,h) Magnified images of gill. Green fluorescence was observed at base of gill filament. (i–k) Magnified images of heart. In some individuals, green fluorescence in part of ventricle. (c,f,i) Bright light images. (e,h,k) Fluorescent images. (d,g,j) Fused images of (c,e), (f,h) or (i,k) respectively. 1, 2, 3, 4, and 5 represent *OlMA1-DsRed2* transgenic male, *OlMA1-GFP* transgenic female, non-transgenic d-rR male, non-transgenic d-rR female, and double transgenic male derived from *OlMA1-DsRed2* transgenic male and *OlMA1-GFP* transgenic female, respectively. 6, 7, 8 and 9 in c indicate gill, liver, gut and spleen, respectively. The color panel in b indicates vivid yellowish green (Munsell color number: 8GY 6.0/11.5), vivid orange (4YR 6.0/14.0), bright purplish red (10RP 4.5/13.5), light orange (8YR 8.5/6.5), and pale beige (8YR 9.0/2.2) from left, respectively.

ijima *et al.*[9] produced transgenic zebrafish strains harboring the *GFP* gene regulated by the regulatory region of the α- and β-*actin* genes, but they did not discuss fluorescence intensity under daylight. Gong *et al.*[13] established transgenic zebrafish strains using the regulatory region of the *zebrafish myosin light chain 2* gene. The fluorescence in skeletal muscle of these strains is so intense that the fish glow green, red, or orange under daylight, thus suggesting their utility as ornamental fish and biomarkers. Chou *et al.*[8] produced transgenic medaka strains with the *GFP* gene regulated by the regulatory region of the zebrafish β-*actin* gene, like Higashijima *et al.*[9] Chou *et al.* observed green fluorescence of their transgenics under daylight.[8] However, the intensity was rather weak compared with those of the transgenics reported here. We produced a transgenic medaka strain with intense green fluorescence in skeletal muscle and eyes using the medaka β-actin regulatory region, but the fluorescence intensity was insufficient for detection with the naked eye.[6,7] The present study used the regulatory region of the *skeletal muscle actin* gene to express the reporter gene. Kusakabe *et al.* reported that the region mimicked the expression pattern of the intrinsic *skeletal muscle actin* gene, judging from whole-mount *in situ* hybridization analysis.[14]

The medaka strains established in the present study were superior to the transgenic strains described above. First, the expression of the fluorescent protein mimicked that of α-actin, indicating that the skeletal muscle was labeled. Second, the fluorescence intensity was high enough to discriminate individuals under daylight without instruments such as a fluorescence microscope. The former is advantageous for studies of muscle development because skeletal muscle can be monitored from the embryo to adulthood without killing the fish. These strains are also helpful for screening cells expressing α-actin, which might be involved in muscle contraction (except in skeletal muscle cells), as shown in Figs 2g and 3d. The latter feature is useful for the study of behavior. Particularly, even individual hatchlings can be identified and traced. High levels of GFP might allow fluorescence detection within a very narrow excitation range, so that behavior can be traced in the dark.

ACKNOWLEDGMENTS

The author thanks Dr T. Kusakabe (Himeji Institute of Technology) for the gift of the plasmid, MA1 (– 1430)intron(+). This study was supported by Grants-in Aid for Scientific Research of Priority Area (1123608) and by the Research for Millennium Project of the Ministry of Education, Culture, Sport, Science and Technology.

REFERENCES

1. Ozato K, Wakamatsu Y. Developmental genetics of medaka. *Dev. Growth Differ.* 1994; 36: 437–443.
2. Tanaka M, Kinoshita M. Recent progress in the generation of transgenic medaka (*Oryzias latipes*). *Zool. Sci.* 2001; 18: 615–622.
3. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. *Science* 1994; 263: 802–805.
4. Kinoshita M, Kani S, Ozato K, Wakamatsu Y. Activity of the medaka translation elongation factor 1α-A promoter examined using the GFP gene as a reporter. *Dev. Growth Differ.* 2000; 42: 469–478.
5. Tanaka M, Kinoshita M, Kobayashi D, Nagahama Y. Establishment of medaka (*Oryzias latipes*) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: A useful model to monitor germ cells in a live vertebrate. *Proc. Natl Acad. Sci. USA* 2001; 98: 2544–2549.
6. Yamauchi M, Kinoshita M, Sasanuma M, Tsuji S, Terada M, Morimyo M, Ishikawa Y. Introduction of a foreign gene into medakafish using the particle gun method. *J. Exp. Zool.* 2000; 287: 285–293.
7. Kinoshita M, Yamauchi M, Sasanuma M, Ishikawa Y, Osada T, Inoue K, Wakamatsu Y, Ozato K. A transgene and its expression profile are stably transmitted to offspring in transgenic medaka generated by the particle gun method. *Zool. Sci.* 2003; 20: 869–875.
8. Chou CY, Horng LS, Tsai HJ. Uniform GFP-expression in transgenic medaka (*Oryzias latipes*) at the F0 generation. *Transgenic Res.* 2001; 10: 303–315.
9. Higashijima S, Okamoto H, Ueno N, Hotta Y, Eguchi G. High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. *Dev. Biol.* 1997; 192: 289–299.
10. Hsiao CD, Hsieh FJ, Tsai HJ. Enhanced expression and stable transmission of transgenes flanked by inverted terminal repeats from adeno-associated virus in zebrafish. *Dev. Dyn.* 2001; 220: 323–336.
11. Higashijima S, Hotta Y, Okamoto H. Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *J. Neurosci.* 2000; 20: 206–218.
12. Ju B, Chong SW, He J, Wang X, Xu Y, Wan H, Tong Y, Yan T, Korzh V, Gong Z. Recapitulation of fast skeletal muscle development in zebrafish by transgenic expression of GFP under the mylz2 promoter. *Dev. Dyn.* 2003; 227: 14–26.
13. Gong Z, Wan H, Tay TL, Wang H, Chen M, Yan T. Development of transgenic fish for ornamental and bioreactor by strong expression of fluorescent proteins in the skeletal muscle. *Biochem. Biophys. Res. Comm.* 2003; 308: 58–63.

14. Kusakabe R, Kusakabe T, Suzuki N. *In vivo* analysis of two striated muscle actin promoters reveals combinations of multiple regulatory modules required for skeletal and cardiac muscle-specific gene expression. *Int. J. Dev. Biol.* 1999; **43**: 541–554.
15. Yamamoto T. Changes of cortical layer of the egg of *Oryzias latipes* at the time of fertilization. *Proc. Imp. Acad. (Tokyo)* 1939; **15**: 269–271.
16. *Clontech*. Living Colors TM DsRed2. *CLONTECHniques*, XVI. Clontech, Palo Alto, CA. 2001; 2–3.
17. Baird GS, Zacharias DA, Tsuen RY. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proc. Natl Acad. Sci. USA* 2000; **97**: 11984–11989.